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Award Number DAMD17-94-J-4421

TITLE: Breast Cancer Research Training Grant

PRINCIPAL INVESTIGATOR: Adrienne E. Rogers, M.D.

CONTRACTING ORGANIZATION: Boston University School of Medicine
Boston, Massachusetts 02118

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Fort Detrick, Maryland 21702-5012

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12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) The purpose of the program is to train predoctoral students at Boston University Schools of Medicine and Public Health (BUSM, BUSPH) in research into the etiology, prevention, detection, diagnosis and therapy of breast cancer using the most advanced knowledge and techniques available. In addition to providing training in the student's chosen discipline, the program ensures her or his education in other relevant disciplines. Emphasis is placed on interdisciplinary training in Pathology, Epidemiology, and Cell and Molecular Biology. The goal is that, upon completion of the degree in a particular discipline, trainees will be able to work and communicate effectively with other scientists in interdisciplinary approaches to breast cancer research. This is being accomplished through an interdepartmental curriculum, selection of research supervisors whose research is in breast cancer or highly relevant to breast cancer and participation in working groups and seminars. Eight trainees, two per year, have been selected on the basis of their GPA, GRE scores, letters of recommendation, interviews, and demonstrated ability in and commitment to research, particularly in breast cancer. All trainees complete a practical course in mammary carcinogenesis studies in rats or mice. Eight have completed their first-year or first two-year courses and are completing laboratory rotations or performing dissertation research; five have passed the qualifying examination. In addition to support from this grant, trainees are supported by tuition and stipend given by BUSM and BUSPH.				
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			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

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NA Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.


NA In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

NA For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

NA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

NA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.


PI - Signature

3/19/99
Date

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BREAST CANCER RESEARCH TRAINING PROGRAM (BCRTP)

5. INTRODUCTION

Information about breast cancer is increasing rapidly from many sources, but much of it is inconsistent and controversial because of the complexity of the disease and its causes. Difficulties arise also in integration of knowledge and understanding and in extrapolating results between and within scientific disciplines. The Program in Research on Women's Health at Boston Medical Center (BMC) has been developed to address some of these problems by serving as an integrating and promoting force for research in women's diseases with a major effort focused in breast cancer research. Through the collaborative efforts in research and teaching already in place and with the stimulus of this Breast Cancer Research Training Program, the work of investigators at BMC is being brought to bear on training students to conduct research on breast cancer.

The **PURPOSE** of the Program was to: 1) establish a formal multidisciplinary research and academic training program in breast cancer biology and epidemiology; 2) produce graduates in one discipline (Pathology, Epidemiology) who have an understanding of the other discipline and who can perform collaborative, multidisciplinary research in the etiology, prevention and therapy of the disease; 3) provide training in cell and molecular biology, experimental pathology, carcinogenesis, epidemiology and biostatistics, immunology, toxicology, and nutrition that will permit trainees to explore: a) basic breast cancer cell processes and interactions, including oncogene regulation, cell signalling, and genetic considerations in design of therapeutic agents; b) questions about etiology, prevention and therapy of breast cancer in laboratory animals and human populations; and c) the integration of knowledge derived from the different approaches; 4) maintain and increase collaborative research in breast cancer and closely related areas among faculty and trainees; 5) provide attractive opportunities for all students and, specifically, for women and underrepresented minorities to pursue careers in breast cancer research. We are promoting the development of young investigators who will have a broad, multidisciplinary background in breast cancer biology and epidemiology and intensive training in a specialized research area

and who can perform significant research using advanced concepts and techniques and communicate research accomplishments effectively. They will be a resource to meet future personnel requirements for breast cancer research.

The **METHODS** are as follows. Doctoral students committed to cancer research with an interest in breast cancer research have been admitted to the Departments of Pathology and Laboratory Medicine or Biostatistics and Epidemiology and have followed a curriculum specifically designed for this Training Program. BC RTP ensures that each predoctoral student: 1) participates in an appropriate, integrated curriculum focused on breast cancer; 2) has an advisory committee, composed of basic science and epidemiology faculty members with expertise in or closely related to breast cancer research; 3) participates actively in seminars, and local, regional and national meetings in addition to informal research meetings at the school.

The students are closely integrated into the Breast Cancer Working Group in the Program in Research on Women's Health. The Group comprises over 50 members in multidisciplinary teams collaborating in breast cancer research and developing new research strategies. The Group stimulates research interactions by providing teaching and discussion of clinical and research topics at monthly meetings. The BC RTP is significantly extending and supplementing the doctoral programs in the participating departments. The BC RTP is directed by Adrienne E. Rogers, MD, and Theodore Colton, DSc, with extensive input from Gail Sonenshein, PhD, and Marianne Prout, MD, who direct the Research on Women's Health Program.

Drs. Rogers and Colton have taken responsibility for all decisions on student admission, performance and training with substantial input from the faculty Trainers and from the Trainees themselves. Because of the relatively small size of the Program and the extensive interactions with faculty in the Women's Health Program, the Admissions and Performance, Recruitment and Seminar Committees proposed have not been needed and, therefore, have not been formally set up.

The trainees were supported annually by the BC RTP and BUSM and BUSPH. The two schools have supplemented the BC RTP so that each student received a stipend of \$15,000-17,000 per year (the level is set by the graduate school each year) and tuition for 20-24 credits per year as needed. Drs. Rogers and Colton reviewed the Program and its requirements and opportunities with the students and answered questions and planned each student's curriculum. Trainees were encouraged to consult any of the participating faculty for general advice

or further discussion of their research interests, and were directed to appropriate faculty by Drs. Rogers and Colton. Trainees have had additional contact with faculty members in courses and seminars.

Drs. Rogers and Colton met also with trainees to discuss their academic performance, to obtain feedback about the program and to advise the trainee on choice of courses and lab rotations. When the students moved into their dissertation research, their Faculty Trainer became their major adviser, but Drs. Rogers and Colton continued to meet individually with them at least once a year. After passing qualifying exams, trainees' research progress has reviewed on a regular basis by their dissertation committees. These committees meet with the trainee at least twice a year, usually starting within six months of the qualifying exam when the student presents her or his research proposal. At subsequent meetings the student gives a progress report. Finally, the committee will serve as the examining committee for the dissertation defense.

6. BODY

The initial application was made to admit and support 4-7 trainees per year, but the grant awarded was for the partial support of 2-4 trainees per year.

The eight students are:

1994: Yvette Cozier (BA, Liberal Arts, Harvard Extension School, 1987; MPH, BUSPH, 1994) was admitted to the Biostatistics and Epidemiology DSc. program. She had extensive laboratory experience in Hematology and in Microbiology (1982-1994), strong letters from faculty and a 3.5 GPA at BUSPH. She was particularly interested in Dr. Lynn Rosenberg's epidemiological studies in breast cancer and other diseases in black women (Black Women's Health Study), and has joined Dr. Rosenberg's group for her dissertation research. She is currently supported by Dr. Rosenberg's research funds. Yvette is an African-American.

Laurie Hafer (BS, Microbiology, Penn. State Univ., 1989) was admitted to the Pathology and Laboratory Medicine Program. She had extensive clinical and research experience in the Immunohistochemistry laboratory at the College of Medicine-University Hospital, Hershey, PA, where she was in charge of research and development with a major focus on breast cancer studies. She had very strong letters from faculty who had supervised and worked with her. Her dissertation research title is "Black tea and estrogen and progesterone receptors in the rat mammary gland". Her work in hormone receptor cellular and molecular biology is with Drs. Rogers and Traish. She has been supported by Dr. Rogers'

research funds and department funds. She participated in the AACR Keystone workshop on Histopathobiology of Neoplasia in July, 1996, and presented a poster there. She was one of ten students selected to give a platform presentation at the national DOD Breast Cancer Meeting, 10/31-11/3/97, in Washington, D.C. and also presented her work at the national Society of Toxicology meeting in March, 1998.

1995. Sylvia Marecki (BS, Microbiology, Univ. N.H., 1995) was admitted by the Pathology and Laboratory Medicine Program and is in the Immunology track. She had significant undergraduate research experience and had been awarded two competitive research grants in addition to a four-year scholarship. She had very strong letters from her research adviser and other faculty. She has completed her course requirements, passed the qualifying exam in June, 1997, and is performing her dissertation research with Dr. Fenton in the regulation of inducible macrophage gene expression at the level of gene transcription. She is supported by Dr. Fenton's research funds and was awarded a research fellowship from the graduate school.

Paul Johansen (formerly Mange) (BS Biology, Yale, 1988) was admitted to the Biostatistics and Epidemiology PhD program. This program differs from the DSc program in the SPH in being a more extensive joint program with the Mathematics Dept. on the Charles River Campus of BU and in requiring a more sophisticated mathematics and biostatistics curriculum and dissertation. After completing 1 1/2 years of medical school at Univ. Mass, Paul left to pursue interests in math and statistics and worked in biomedical applications of these areas as Sr. Research Analyst in Psychiatric Epidemiology at the Mass. General Hospital. He had excellent letters from faculty and colleagues. Paul completed his courses, passed his qualifying examination and is performing his dissertation research with Dr. Timothy Rebbeck at the Univ. of Pennsylvania. His title is "Breast and ovarian cancer risk assessment following prophylactic surgery: A comparison of statistical models using BRCA1 and BRCA2 pedigree data."

1996 Jackie Ashba (BA, Biology & Economics, Clark University, 1989; MPH, BUSPH, 1992; MA, BUSM, Medical Sciences, 1994) was admitted to the Epidemiology DSc program. Her Masters degree research included studies of both biological and epidemiological aspects of breast cancer focusing on hormone receptor phenotypes in breast cancer. Her academic record here and her letters of recommendation are excellent. She has completed her courses and is preparing for the qualifying exam and investigating several possibilities for dissertation research.

Ingrid Gherson (BS, Biology, Binghamton University SUNY) was admitted to the Dept. of Pathology and Laboratory Medicine Program. Her excellent undergraduate record (GPA 3.5) and GRE scores, strong letters of recommendation and significant technical experience in a pathology laboratory all were evidence of her potential for success in the program. She has completed her courses, passed the qualifying exam and begun dissertation research on effects of vitamin D analogues on breast cancer cells with Dr. Michael Holick.

1997 Kathryn Kavanagh(MD-PhD student at BUSM), was admitted to the Department of Pathology and Laboratory Medicine as an MD-PhD student from the Royal College of Surgeons, Dublin, and following a year of molecular biology research at Albert Einstein College of Medicine. Kathryn has a 3.5 GPA here, has completed her course work passed the qualifying exam and is working with Dr. Gail Sonenshein on studies of the inhibitor of NF-KB, I κ B- α , in breast cancer. She was awarded a Grunebaum Cancer Research Fellowship by the Medical School to continue her research.

Elizabeth Jiyoung Lee(BS, Microbiology and Genetics, UCLA 1997). Elizabeth was admitted to the Department of Pathology and Laboratory Medicine Program with excellent GRE scores and experience in studies in laboratory rodents; her research mentors wrote very strong letters. She complete her course work and began dissertation research with Dr. David Sherr on the Ah receptor response to DMBA in mammary gland epithelial and other cells. However, she withdrew from the program at the end of the last term because she was questioning her motivation to pursue a research career.

The BC RTP students participated actively in the setting up and running of DMBA mammary tumorigenesis studies under Dr. Rogers' direction. They learned basic methods for such studies, participated in feeding, weighing and observing the animals and in performing necropsies for examination of tumors and collection of tissues for histological, endocrine and molecular studies. Members of the Pathology faculty introduced them to clinical studies of breast cancer. The subjects specifically covered were: Dr. De las Morenas: basics of breast cancer pathology; Dr. Burke: basics of image analysis, focused on estrogen receptor assay; Dr. Yang and one of the students, Laurie Hafer: basics of immunohistochemistry staining and interpretation; Dr. Rogers: basics of the histopathology of rat mammary gland tumors and discussion of recent research papers.

The students participated in both the Research in Women's Health and the Pathology seminar series and in Breast Cancer Working Group meetings in addition to a variety of other seminars in the two schools and in the Mass. Breast Cancer Research Program.

7. CONCLUSIONS

The Program has actively recruited, attracted and retained excellent students from diverse backgrounds to focus on breast cancer research. **Of the eight students supported, five are actively engaged in breast cancer research as the major focus of their dissertation; one is engaged in basic gene transcription studies related to cytokines that are applicable to breast cancer research; one, already experienced in breast cancer research, is currently deciding on her research program; one has left the graduate program for motivational, not academic, reasons.** Therefore, the program has provided a very high yield of young breast cancer investigators in biological or epidemiological research. The students are a cohesive group who study and work together well. There have been productive discussions of data from the DMBA project within the group that foster the interdisciplinary goals of the program. The Trainees interacted extensively with Drs. Rogers & Colton and with other students and faculty working in breast cancer research and working in clinical settings with breast cancer. They are doing well in course work and research. The interdisciplinary focus is strong, fostered by the practical course, the required epidemiology and pathology courses, seminars, and frequent formal and informal meetings of the students with Drs. Rogers and Colton. The students are progressing as expected (or more rapidly than expected) through their course work and into research, a commendable result.

8. REFERENCES

None

**9. APPENDICES
(SEMINARS)**



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

BREAST CANCER WORKING GROUP

Meeting Announcement

REGULATION OF p53 FUNCTION DURING NORMAL AND NEOPLASTIC MAMMARY DEVELOPMENT

Presented by

D. JOSEPH JERRY, Ph.D.

**Department of Veterinary and Animal Sciences
University of Massachusetts, Amherst**

Monday, December 7, 1998

4:00 PM

R-115



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

HEMATOLOGY/ONCOLOGY RESEARCH SEMINAR

Dr. Phillipe Anker

of the

UNIVERSITY OF GENEVEA

will speak on

**Circulating tumor DNA in the plasma of
breast cancer patients**

TUESDAY, NOVEMBER 17, 1998

4 PM

E-518



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

BREAST CANCER WORKING GROUP

Meeting Announcement

BREAST CANCER PREVENTION TRIALS:

RESULTS AND QUESTIONS

Presented by

MARIANNE N. PROUT, M.D., M.P.H.

**Department of Surgery
School of Public Health
BUMC**

Monday, October 26, 1998

4:00 PM

R-115



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

in conjunction with
The Department of Biochemistry

HORMONAL CONTROL OF BRANCHING MORPHOGENESIS IN THE MOUSE MAMMARY GLAND

by

DR. BARBARA K. VONDERHAAR, Ph.D.

**Chief, Molecular and Cellular Endocrinology Section
Laboratory of Tumor Immunology and Biology/NCI**

Tuesday, February 10, 1998

12:00 PM

L-112

Refreshments will be served at 11:45 AM



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

in conjunction with

**MASSACHUSETTS DEPARTMENT OF PUBLIC HEALTH
BREAST CANCER RESEARCH PROGRAM**

presents

**Gradual Epigenetic Conversion to
Immortality and Telomerase Expression in
Cultured Human Mammary Epithelial Cells**

by

Dr. Martha Stampfer

**Senior Staff Scientist
Lawrence Berkeley National Laboratory
University of California**

Wednesday, January 28, 1998

Bakst Auditorium

4:00 PM

Refreshments will be served

BREAST CANCER WORKING GROUP

Meeting Announcement

**BREAST CANCER ON CAPE COD:
HYPOTHESES AND UNCERTAINTIES**

Presented by

DR. RICHARD CLAPP, Sc.D.

**Environmental Health
Boston University School of Public Health**

**Thursday, December 4, 1997
4:00 PM
R-103**

Refreshments will be served

BREAST CANCER WORKING GROUP

MEETING ANNOUNCEMENT

Role of Cell Cycle Regulators in Carcinogen- Induced Rat Mammary Tumors

presented by

DR. JIM XIAO

Departments of Medicine and Biochemistry

Thursday, November 20, 1997

4:00-5:00 PM

R-108

Refreshments will be served



Program in Research on Women's Health

Interdisciplinary Seminar Series

Current Directions in Research on Women's Health

BREAST CANCER WORKING GROUP

MEETING ANNOUNCEMENT

Role of Cell Cycle Regulators in Carcinogen-Induced Rat Mammary Tumors

presented by

DR. JIM XIAO

Departments of Medicine and Biochemistry

Thursday, November 13, 1997

4:00-5:00 PM

R-108

Refreshments will be served

Tuesday, November 3, 1998

CANCER PREVENTION & CONTROL GRAND ROUNDS

**McNary R103
12:30 PM to 1:30 PM**

*"Breast Cancer Prevention...
The New Era Has Begun"*

**Hear
the results
of the landmark
Breast Cancer
Prevention
Trial**

Dr. Maureen Kavanah
National Protocol Chair

Dr. Ted Colton
Chair of the External Review and
Safety Monitoring Advisory Committee

Dr. Marianne Prout
Principal Investigator, Moderator
Boston Medical Center

**Lunch provided
1.2 Nursing CEUs/Physician CMEs**

**BOSTON
MEDICAL**
CENTER

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CANCER PREVENTION & CONTROL GRAND ROUNDS

McNary Learning Center, R103 ■ 12 Noon to 1 PM

Tuesday, January 13, 1998

"PCBs in Massachusetts: Is there a Cancer Risk?"

Richard Clapp, ScD, Associate Professor of Public Health, Department of Environmental Health and Marianne Prout MD, MPH (Discussant)

Tuesday, February 10, 1998

"Nutrition and Cancer Prevention: What do Clinicians need to know?"

Walt Willett MD, DrPh, Professor of Epidemiology and Nutrition, Harvard School of Public Health, Chairman, Department of Nutrition

Tuesday, March 10, 1998*

"Cancer in the Black Women's Health Study"

Lynn Rosenberg, ScD, Research Professor, Boston University School of Medicine and Public Health, Department of Epidemiology and Biostatistics, and Assistant Director, Slone Epidemiology Unit

Tuesday, April 14, 1998

"Early Detection and Screening for Melanoma"

Thomas Rohrer, MD, Assistant Professor, Dermatology and Alan Geller, RN, MPH, Research Assistant Professor, Dermatology and Epidemiology/Biostatistics (SPH)

Tuesday, May 12, 1998*

"Cervical Cancer Screening: The Curable Cancer not yet Conquered"

Karen Freund, MD, MPH, Associate Professor of Medicine and Public Health and Valena Soto-Wright, MD, Director of Gynecologic Oncology, Assistant Professor of Obstetrics and Gynecology

*March 10 and May 12 programs are jointly sponsored by the Center of Excellence in Women's Health

■ Physician CME's provided ■ Lunch Provided

The logo for Boston Medical Center, featuring the words "BOSTON MEDICAL" in a bold, sans-serif font, with "CENTER" in a smaller font above "MEDICAL". A circular graphic element is partially visible behind the text.

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Tuesday, May 12, 1998

CANCER PREVENTION & CONTROL GRAND ROUNDS

**Keefer Auditorium
12 Noon to 1 PM**

“Cervical Cancer Screening: The Curable Cancer Not Yet Conquered”

Karen Freund, MD, MPH

Associate Professor of Medicine and Public Health, BUSM

Valena Soto-Wright, MD

Director of Gynecologic Oncology, BMC

Assistant Professor of Obstetrics and Gynecology, BUSM

Lunch provided/Physician CME's provided

*Program is jointly sponsored by the
Center for Excellence in Women's Health*

**BOSTON
MEDICAL**
CENTER

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Tuesday, March 10, 1998

CANCER PREVENTION & CONTROL GRAND ROUNDS

**McNary Learning Center, R103
12 Noon to 1 PM**

"Cancer in the Black Women's Health Study"

Main speaker

Lynn Rosenberg, ScD

Research Professor, Department of Epidemiology and Biostatistics
Boston University School of Medicine and Public Health

Assistant Director, Slone Epidemiology Unit

*Program jointly sponsored by the
Center of Excellence in Women's Health

Lunch provided/Physician CME's provided



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Tuesday, February 10, 1998

CANCER PREVENTION & CONTROL GRAND ROUNDS

**McNary Learning Center, R103
12 Noon to 1 PM**

*“Nutrition and Cancer Prevention:
What do Clinicians need to know?”*

Main speaker

Walter Willett MD, DrPh
Professor of Epidemiology and Nutrition
Chairman, Department of Nutrition
Harvard School of Public Health

Lunch provided/Physician CME's provided



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Massachusetts Department of Public Health

Breast Cancer Research Program

1997-1998 Lecture Series

Date/Time	Speaker	Location	Topic
October 3, 1997 3:30pm	Judah Folkman, MD Professor of Cell Biology Harvard Medical School	Brigham & Women's Hospital Bornstein Family Amphitheater	"Angiogenesis in Breast Cancer"
November 6, 1997 4:00pm	Emil Frei III, MD Physician-in-Chief Emeritus Dana-Farber Cancer Institute	Dana-Farber Cancer Institute Smith Family Room Boston, MA	"Treatment of Metastatic Breast Cancer"
December 9, 1997 3:30pm	Karen Freund, MD, MPH Associate Professor of Medicine Chief, Women's Health Unit Boston University School of Medicine	Boston University Medical School Bakst Auditorium	"Physician - Patient Communication"
January 28, 1998 4:00pm	Martha R. Stampfer, Ph.D. Senior Staff Scientist Lawrence Berkeley National Laboratory, University of California	Boston University Medical School Bakst Auditorium	"Gradual Epigenetic Conversion to Immortality and Telomerase Expression in Human Mammary Epithelial Cells"
February 11, 1998 12:00noon	Matthew P. Longnecker, MD, Sc.D. National Institute of Environmental Health Sciences- Epidemiology Branch Research Triangle Park, North Carolina	Harvard School of Public Health RHS Bldg. Room G-12 (basement) Huntington Avenue, Boston	"Alcohol and Breast Cancer"

Date/Time	Speaker	Location	Topic
February 27, 1998 12 noon	Steven Narod, MD Chair, Breast Cancer Research Ontario Cancer Institute Ontario, Canada	Harvard Institutes of Medicine First Floor Conference Room #109 Boston, MA	"Genetic Testing and BRCA 1"
March 9, 1998 2:30pm	Devra Davis, MD Director, Health, Environment, and Development Program World Resources Institute Washington, DC	University of Lowell Alumni Lounge Lyden Library North Campus	"Rethinking Risk Factors for Breast Cancer: Why the Environment Matters
April 8, 1998	Annual Breast Cancer Research Symposium	Holiday Inn 5 Blossom Street Boston, MA	1997 Breast Cancer Research Awardees present
May 5, 1998	Lewis A. Chodosh, MD, PhD University of Pennsylvania Medical School, Department of Molecular and Cellular Engineering	Dana-Farber Cancer Institute	"Mammary Gland Development, Reproductive History and Breast Cancer Risk
May 22, 1998 11:00am	Michael T. Lotze, MD University of Pittsburgh Professor of Surgery/ Molecular Genetics and Biochemistry Division of Surgical Oncology and Biotherapy	University of Massachusetts Medical Center Worcester, MA Gift Learning Center Auditorium S1-608	"Into Thin Air: Apoptosis and Survival of Immune Effectors Within the Tumor Microenvironment"

Please call Kris Lyons at 617-624-5466 for more information.

Department of **PATHOLOGY** and Laboratory Medicine



Boston University School of Medicine, 715 Albany Street, Boston, MA 02118-2394

PATHOLOGY SEMINARS, SPRING TERM 1999

FRIDAYS, 1:45-2:45, ROOM L301, REFRESHMENTS AT 1:30

FEBRUARY

- | | | |
|----|--|--|
| 12 | Dr. Howard Eichenbaum
Department of Psychology, Boston University | <i>Neural mechanisms of declarative memory.</i> |
| 19 | Dr. Arthur Sytkowski
Beth Israel Hospital | <i>Selenium and prostate cancer.</i> |
| 26 | Dr. David Cameron
Department of Physiology
Boston University School of Medicine | <i>Structural and physiological aspects of retinal regeneration.</i> |

MARCH

- | | | |
|----|--|---|
| 5 | Dr. William Greenlee
Department of Pharmacology & Molecular Toxicology
University of Massachusetts Medical School | <i>Dioxin: poison or probe? Insights into a novel mechanism coupling cell cycle regulation with gene transcription.</i> |
| 12 | ****SPRING BREAK**** | |
| 19 | Dr. Ulla Hansen
Biology Department, Boston University | TBA |
| 26 | Dan Maravei
Department of Pathology & Laboratory Medicine | <i>Genetic rescue of ovarian failure.</i> |

APRIL

- | | | |
|----|--|--|
| 2 | Terry Means
Department of Pathology & Laboratory Medicine | <i>LAM and LPS signaling requires different toll-like receptors.</i> |
| 9 | Dr. Wilma Wasco
Department of Neurology
Harvard Medical School/Massachusetts General Hospital | <i>Genes associated with familial Alzheimer's Disease.</i> |
| 16 | Dr. Cynthia Morton
Departments of Obstetrics, Gynecology and Reproductive Biology and Pathology
Brigham & Womens Hospital | <i>Many genes and many tumors: Genetics of uterine leiomyomata.</i> |
| 23 | Dongpo Cai
Department of Pathology & Laboratory Medicine | <i>Functional study of AND34, a novel p130 cas-binding protein.</i> |
| 30 | Dr. Tom McGarry
Beth Israel Hospital/Harvard Institute of Medicine | <i>Geminin: A novel inhibitor of DNA replication.</i> |

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PLEASE POST
Phone: 638-4500

Department of **PATHOLOGY** and Laboratory Medicine



Boston University School of Medicine, 715 Albany Street, Boston, MA 02118-2394

PATHOLOGY SEMINARS, FALL TERM 1998
FRIDAYS, 1:45-3:00, ROOM L301, REFRESHMENTS AT 1:30

OCTOBER

- | | |
|--|---|
| <p>2 Dr. Alex Toker
<i>Boston Biomedical Research Institute</i></p> <p>9 Dr. Dana Gabuzda
<i>Harvard Medical School</i></p> <p>16 Ryan Pavlovich
<i>Department of Pathology & Laboratory Medicine</i></p> <p>23 Sylvia Marecki
<i>Department of Pathology & Laboratory Medicine</i></p> <p>30 Dr. Marian DiFiglia
<i>Harvard Medical School</i></p> | <p><i>Signaling through PI-3 kinase.</i></p> <p><i>Chemokine receptors and mechanisms of cell death in HIV infection of the CNS.</i></p> <p><i>IL-15 signaling and function in murine macrophages.</i></p> <p><i>The role of interferon-regulated factors (IRF) ICSBP and IRF-4 in macrophage activation.</i></p> <p><i>New perspectives on the pathogenesis of Huntington Disease.</i></p> |
|--|---|

NOVEMBER

- | | |
|--|---|
| <p>5 Dr. William Kaelin
<i>Howard Hugues Medical Institute</i>
<i>Dana Farber Cancer Institute</i>
<i>Harvard Medical School</i></p> <p>13 Dr. Jon D. Goguen
<i>University Mass Medical School</i></p> <p>20 Dan Maravei
<i>Department of Pathology & Laboratory Medicine</i></p> | <p><i>Function of the Von Hippe-Lindau tumor suppressor gene.</i></p> <p><i>Plasminogen deficient mice have increased resistance to plague.</i></p> <p><i>Genetic rescue of germ cell apoptosis as caused by disruption of the gene mutated in ataxia-telangiectasia (atm) in Mice.</i></p> |
|--|---|

27 THANKSGIVING HOLIDAY

DECEMBER

- | | |
|---|---|
| <p>4 Elisabetta Del Re
<i>Department of Pathology & Laboratory Medicine</i></p> <p>11 Dr. Stuart Tobet
<i>Eunice Kennedy Shriver Center</i>
<i>for Mental Retardation</i></p> | <p><i>Altered cathepsin H expression patterns in colorectal cancers correlate with cancer stage and site.</i></p> <p><i>Regulation of cellular organization in developing neuroendocrine brain.</i></p> |
|---|---|

Boston University School of Medicine is accredited by the Accreditation Council for Continuing Medical Education to sponsor continuing medical education for physicians. Boston University School of Medicine designates this educational activity for a maximum of (#of hours) hours in category 1 towards the AMA Physician's Recognition Award. Each physician should claim only those hours of credit that he/she actually spent in the educational activity.

PLEASE POST
Phone: 638-4500

Department of **PATHOLOGY** and Laboratory Medicine



Boston University School of Medicine, 715 Albany Street, Boston, MA 02118-2394

PATHOLOGY SEMINARS, SPRING TERM 1998
FRIDAYS 1:00-2:30, ROOM L301, REFRESHMENTS AT 1:00

FEBRUARY

- 6 Driss Zoukhri, Ph.D.**
Schepens Eye Research Institute, Boston
Is the neural regulation of the lacrimal gland altered in Sjögren's syndrome
- 13 Elizabeth Battinelli**
Department of Pathology & Laboratory Medicine
Nitric oxide induces apoptosis in human megakaryocytes.
- 27 Katya Ravid, Ph.D.**
Associate Professor, Department of Biochemistry
Mechanisms of action of a ploidy- and proliferation-promoting factor in the marrow: Thrombopoietin.

MARCH

- 6 Gerald P. Bailey**
Department of Pathology & Laboratory Medicine
Contribution of glutamate receptors to spontaneous and stimulus-evoked discharge patterns in afferent fibers innervating hair cells.
- 13 HOLIDAY**
- 20 HENRY I. RUSSEK DAY**
- 27 Adrian Zai**
Department of Pathology & Laboratory Medicine
The role of surface-membrane PDI in transnitrosation.

APRIL

- 3 Cynthia Lemere, Ph.D.**
Assistant Professor, Harvard Medical School,
Center for Neurological Diseases
Temporal deposition of amyloid and amyloid-associated proteins in an Alzheimer's disease transgenic mouse model.
- 10 Rachael Neve, Ph.D.**
Harvard Medical School, McLean Hospital
A new signal transduction pathway in Alzheimer's disease.
- 17 Harold Chapman, M.D.**
Associate Professor, Harvard Medical School
Cysteine proteases in MHC class II antigen presentation: Lessons from knockout mice.
- 24 Daniel Gaposchkin**
Department of Pathology & Laboratory Medicine
The use of somatic cell mutants in determining regulation of omega-3 fatty acid levels.

MAY

- 1 Gregory Viglianti, Ph.D.**
Associate Professor, Department of Microbiology
Mechanisms of retinoic acid and interleukin-16-mediated repression of HIV-1 transcription.
- 8 James Liou**
Department of Pathology & Laboratory Medicine
Molecular mechanisms of ras-mediated apoptosis.

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PLEASE POST
Phone: 638-4500

Slone Memorial Lecture

*"Diet, Genetic Polymorphisms, and
Breast Cancer"*

Lucile Adams-Campbell, Ph.D.

Director, Howard University Cancer Center

Wednesday, March 25, 1998

Noon - 1 p.m.

*Slone Epidemiology Unit
Third Floor Conference Room
1371 Beacon Street
Brookline, MA 02146*

For further info., call Richard Vezina or Sowmya Rao at (617) 734-6006

Hematology-Oncology

RESEARCH ROUNDTABLE

Topic: Aryl-hydrocarbons, Cell Cycle Checkpoints and Carcinogenesis

Presented by: Cyrus Vaziri,
Cancer Center, BUSM

Abstract:

Polycyclic Aryl-hydrocarbons (PAHs) are abundant and ubiquitous environmental pollutants with well-documented mutagenic and carcinogenic properties. Cytochrome P450-mediated oxidation of PAHs generates electrophilic metabolites which form covalent adducts with genomic DNA. Error-prone replication of abducted DNA can result in frame-shift and deletion mutations. Such PAH-induced mutations in proto-oncogenes or tumor suppressor genes are thought to contribute to malignant transformation. Thus, a crucial requirement for PAH-induced carcinogenesis is that PAH-damaged bases become mis-replicated during DNA synthesis. We have identified a cell cycle checkpoint resulting from PAH-induced DNA damage which arrests proliferating cells in G1. This checkpoint prevents mutagenic mis-replication of damaged DNA and represents a potentially important mechanism for guarding against PAH-induced mutagenesis and transformation. Therefore, we seek to elucidate the molecular basis of PAH-induced cell cycle checkpoint control. In preliminary studies we have shown that PAH-induced G1 arrest is p53 and p21-independent. The p53/p21-independence of PAH-induced G1 arrest distinguishes this checkpoint from those which are elicited by other forms of DNA damage. In order to investigate mechanism of checkpoint control we have tested the PAH sensitivity of mitogenic signaling events during the G1 phase of cell cycle. Our preliminary results suggest a role for the Retinoblastoma (Rb) protein in mediating PAH-induced G1 arrest. Possible mechanism whereby the PAH-induced checkpoint is imposed upon cells, and how the checkpoint may be overcome during chemical carcinogenesis, will be discussed.

Tuesday
November 25, 1997
Evans 518 Conference Room
@ 4:00pm



Boston University School of Medicine

715 Albany Street
Boston, Massachusetts
02118-2526

Instruction in the Responsible Conduct of Research at Boston University School of Medicine

The plan for formal instruction in The Responsible Conduct of Research is a product of a committee of senior faculty and administrators. The Advisory Committee consists of: David Beller (Medicine), Leonard Glantz, (School of Public Health), Conan Kornetsky (Psychiatry), Wayne Lamorte (Surgery), Susan Leeman (Pharmacology), Norman G. Levinsky (Associate Provost), and Mary Williams (Pulmonary Center). From year to year, other senior faculty members participate in planning. The Committee is staffed by Peter Reich, Assistant to the Dean and Director of Special Projects, School of Medicine, to whom inquiries should be directed (617-638-5302 or preich@bu.edu). Student representatives are: Caroline Fisher, Milissa Kaufman, James Chen, Susan Ward.

The plan calls for a series of lectures, seminars and workshops on several major issues. To a very large degree, these topics match those identified by Public Responsibility in Medicine and Research (PRIM&R), the Association of American Medical Colleges and National Institutes of Health. They include Human Subjects, Laboratory Animals, Laboratory Notebooks, Responsibility in Authorship, Institutional Policies on Scientific Misconduct, Proper Application of Statistical Analysis, and Conflict of Interest. Attendance at these sessions is required of pre- and post-doctoral trainees and is strongly recommended for M.D.-Ph.D., and Master Degree candidates. Sign-in sheets from these various programs (1991-1998) are available in the Dean's Office. In addition, most departments run their own training programs to acquaint students with policies and procedures in the laboratory.

The seminar/workshop/lecture series is offered periodically throughout the academic year to provide an ongoing dynamic forum for the exchange of ideas. Certain key lectures are videotaped and offered for use by departments. For example, an institutionally produced videotape on proper care and use of laboratory animals is made available to the training directors. In addition, the NIH videotape on Institutional Review Boards produced by NIH is available. Certain documents such as the University's policies on scientific misconduct and conflict of interest, as well as NSF's On Being a Scientist are available from the Dean's Office. With videotapes and discussion guides available, departments are in a position to enhance instruction offered at the departmental level.

Concurrent with the lecture series, the Advisory Committee explores other ways to present the subject matter and related issues in a manner that reinforces and supports the existing instruction in the proper conduct of research as carried out by training directors and departments.

Copies of announcements for the series 1991-1998 are attached.



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P R E S E N T T H E 1 9 9 9 S E R I E S O N

the RESPONSIBLE CONDUCT *of* RESEARCH

The 1999 Series

Box Lunch in Hiebert Lounge, 12-1 pm

Thursday, February 18

The Boston University Policy on Scientific Misconduct

Introduction: Norman G. Levinsky, M.D., *Professor of Medicine, Associate Provost*

Policy: Michael B. Rosen, *Associate General Counsel, Boston University*

Tuesday, March 30

High Crimes and Misdemeanors: Collecting and Interpreting Data: Part I

Thomas L. Rothstein, M.D., Ph.D.

Professor, Departments of Medicine and Microbiology; Director, Immunobiology Unit

Thursday, April 15

Collecting and Interpreting Data: Part II

Small group discussions based on problems identified on March 30.

Members of the Committee on the Responsible Conduct of Research (faculty and students/trainees) and guests from Basic Science or Clinical Departments will meet with small groups of graduate students/trainees to follow up on the March 30th session and to identify subjects and topics for future sessions.

Attendance is required of all Pre- and Post- doctoral trainees and M.D.-Ph.D. and M.A. candidates.



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PRESENT THE 1998 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

REPRODUCIBILITY IN RESEARCH: *Is One Talking Dog Significant?*

Introduction: Teaching the Dog to Talk

Conan Kornetsky, Ph.D.

Professor of Psychiatry and Pharmacology

Sample Size: Biological vs. Statistical Significance

Timothy C. Heeren, Ph.D.

Associate Professor of Public Health and Pediatrics

N of 1: Drawing Conclusions from Few Observations

Presented by students in the Division of Graduate Medical Sciences:

Andrew Bremer, Milissa Kaufman, Helen Lyons

Presentation Issues for Molecular Biology

Judith A. Foster, Ph.D.

Professor of Biochemistry; Director, DNA Protein Core Facility

Thursday, April 23, 1998

3:30-5:00 pm

Room L-110

Attendance is required of all Pre- and Post- doctoral trainees and M.D.-Ph.D. and M.A. candidates.



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PRESENT THE 1997 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

ARE ANIMALS JUST ANOTHER SUPPLY ITEM? *Issues in the Responsible Use of Animals in Research*

Case 1:

Patients sometimes develop renal failure after receiving contrast dyes during angiography. Investigators at BMC believe that a new prostaglandin analog (Prosta-P) may prevent this by increasing renal blood flow. They want to test this by inducing partial renal failure in 30 dogs by removing all of one kidney and half of the other. One week later they will re-anesthetize the dogs and give an intravenous infusion of contrast. Half of the dogs will receive Prosta-P and half will receive a placebo. They will then follow the dogs to see if Prosta-P lessens the incidence or severity of renal failure.

Case 2:

A biotech company has a patent for a new class of drugs known as "neoamines," and some of these seem to inhibit tumor growth. As they synthesize new compounds, they want to screen them for tumor inhibition potential in nude mice. In collaboration with BMC faculty they want approval to use 25,000 mice over the next 5 years. They plan to inject nude mice with cell lines for breast, colon, and squamous cell cancers and test the ability of neoamines to inhibit tumor growth.

Issues:

When is the use of animals in research justified?

What alternatives should be considered?

What is pain and distress? How do you identify pain/distress in an animal?

What are the researcher's obligations?

How does one estimate and justify the numbers of animals needed?

Panelists

Douglas L. Rosene, Ph.D., Associate Professor of Anatomy and Neurobiology, BUMC

Colleen Cody, Coordinator, Institutional Animal Care and Use Committee, BUMC

Alicia Karas, D.V.M., Assistant Professor of Anesthesiology, Tufts University School of Veterinary Medicine

Moderator

Wayne LaMorte, M.D., Ph.D., M.P.H., Associate Professor of Surgery and Public Health,
Chairman, Institutional Animal Care and Use Committee, BUMC

Thursday, November 20, 1997

3-4:30 pm

Bakst Auditorium

Attendance is required of all Pre- and Post- doctoral trainees and M.D.-Ph.D. and M.A. candidates.



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PRESENT THE 1997 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

IS THIS MY BODY, OR WHAT?

*When and Under What Conditions
May Human Tissue Be Used in Research?*

The Context:

Norman G. Levinsky, M.D.

Professor of Medicine and Associate Provost, Boston University Medical Campus
Editor of *Xenotransplantation: Science, Ethics and Public Policy*.

The Realm:

Leonard Glantz, J.D.

Professor of Public Health and Socio-Medical Sciences and Community Medicine
Co-author of *Informed Consent to Human Experimentation: The Subject's Dilemma* and
Children as Research Subjects: Science, Ethics & Law

The Rules:

John Bernardo, M.D.

Associate Professor of Medicine; Chairman, Boston Medical Center Institutional Review Board

The Case:

The facts of a lawsuit involving human tissue used in research will be distributed at the door.

Wednesday, October 15, 1997

3-4:30 pm

Room L-110

and please mark your calendars for...

Thursday, November 20 at 3 pm

for a session on laboratory animals presented by
the Institutional Animal Care and Use Committee (IACUC)

Attendance is required of all Pre- and Post- doctoral trainees and M.D.-Ph.D. and M.A. candidates.



PRESENT THE 1997 SERIES ON

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the RESPONSIBLE CONDUCT *of* RESEARCH

PROBLEMS IN PROFESSIONAL ETHICS: Avoiding Scientific Plagiarism

"I Couldn't Have Said It Better Myself"

Mary Williams, Ph.D.

Professor of Medicine and Anatomy and Neurobiology,

Co-Editor of *The American Journal of Respiratory Cell and Molecular Biology*, 1988-1993

"Original, With the Minimum of Alteration"

Christopher Ricks, M.A.

Professor of English, Boston University; Author of *T.S. Eliot and Prejudice* and *Beckett's Dying Words*;

Co-editor, *The State of the Language*

Panel Discussion Based on Case Studies

Joseph Loscalzo, M.D., Ph.D.

Professor and Chairman, Division of Medicine; Physician-in-Chief, Boston Medical Center,

Associate Editor, *The New England Journal of Medicine*

Christopher Ricks, M.A.

Johanna vanderSpek, Ph.D.

Assistant Research Professor of Medicine; Chair, Institutional Biosafety Committee

Susan Ward

Ph.D. Candidate, 1998, Behavioral Neurosciences Program

Mary Williams, Ph.D.

Moderator

Room L-110

3:30 - 4:45 pm

Thursday, February 27, 1997

Attendance is required for pre- and post-doctoral trainees.

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PRESENT THE 1996 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

RIGHTS AND RESPONSIBILITIES IN WORKING WITH SCIENTIFIC DATA

Material and Intellectual Transfer between Institutions:

What can you take when you leave?

David Seldin, M.D.-Ph.D.

Assistant Professor of Medicine, Boston University School of Medicine

Narrowing the Gray Area:

Rights to Data, Biological Material and Patentable Inventions

Susan H. Frey, J.D.

Associate General Counsel, General Counsel's Office, Boston University

Patents and Material Transfer Agreements

Ashley Stevens, Ph.D

Director, Office of Technology Transfer, Boston University School of Medicine

A Philosophical Perspective on Ethical Conduct Between Scientists

Alfred Tauber, M.D.

Professor of Medicine, Professor of Philosophy,

Director, Boston University Center of Philosophy and the History of Science

Moderator:

Mary Williams, Ph.D.

Professor of Medicine and Anatomy and Neurobiology

Wednesday, April 24, 1996

Keefer Auditorium

9:00-11:00 am



PRESENT THE 1996 SERIES ON

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the RESPONSIBLE CONDUCT *of* RESEARCH

Basic Rules and Policies

Research Involving Human Subjects

Leonard Glantz, J.D.

Professor of Public Health and Socio-Medical Sciences and Community Medicine, Co-Author of *Informed Consent to Human Experimentation: The Subject's Dilemma* and of *Children as Research Subjects: Science, Ethics & Law*.

The Boston University Policy on Scientific Misconduct

Michael Rosen, J.D.

Associate General Counsel, Boston University; Co-Author, *Boston University's Policies and Procedures Concerning Allegations of Misconduct in Scholarship and Research*.

The Scientific Use of Animals: A Changing Arena

Special Guest Speaker

Peter Theran, V.M.D.

Vice-President, Hospital Division and Director of Laboratory Animal Welfare,
Massachusetts Society for the Prevention of Cruelty to Animals/American Humane Education Society.
Member, National Research Council Committee for Long Term Care of Chimpanzees in Research.
Director of BUSM Laboratory Animal Science Center, 1966-1989.

Wednesday, September 18, 1996

3-5 pm in Room L-110

Attendance is required for pre- and post-doctoral trainees.

and please mark your calendars for..

*Wednesday, October 23, 3-5pm in L-110
for a Presentation on Case Studies in Research Ethics*



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PRESENT THE 1995 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

Presented in Cooperation with Boston University School of Public Health as a Public Health Forum

Disclosure of Potential Conflicts of Interest by Researchers

A discussion of proposed federal regulations and
their impact on biomedical researchers.

led by

Michael Rosen, J.D., M.A.

Associate General Counsel

Boston University



Tuesday, April 4, 1995

4 - 5 pm in Room L-112

Attendance is required for pre- and post-doctoral trainees and M.D. Ph.D. students.



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PRESENT THE 1995 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

Presented in Cooperation with Boston University School of Public Health as a Public Health Forum

Who Gets Authorship and Why?

A presentation of principles that should guide decision-making about authorship and proposed procedures for applying them, followed by a discussion.

Tuesday, February 21, 1995
4-5 pm in Room L-112



Prepared and presented by the Ad Hoc Committee on Authorship,
Boston University School of Public Health:

Wendy Mariner, J.D., M.P.H., *Professor of Public Health and Socio-Medical Sciences
and Community Medicine, Chair.*

Janet M. Lang, Ph.D., Sc.D., *Associate Professor of Public Health.*

Barbara Millen Posner, Dr.P.H., R.D., *Associate Professor of Public Health and
Socio-Medical Sciences and Community Medicine.*

David Sherr, Ph.D., *Professor of Public Health and Pathology and Laboratory Medicine.*

Attendance is required for pre- and post-doctoral trainees.

...and please make a note:

as part of this continuing series on The Responsible Conduct of Research,
The Boston University Policy on Conflict of Interest
will be presented by

Michael J. Rosen, J.D., *Assistant General Counsel, Boston University*
on Tuesday, April 4, 1995, 4-5 pm in Room L-112.

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PRESENT THE 1995 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

Presented in Cooperation with Boston University School of Public Health as a Public Health Forum

Integrity in Research and Scholarship: Who Shall Guard the Guardians?

Judith P. Swazey, Ph.D.

President, The Acadia Institute, Bar Harbor, Maine

Adjunct Professor, Social and Behavioral Sciences, Boston University School of Medicine

Tuesday, November 29, 1994

4-5 pm in Room L-112

Dr. Swazey will examine two interrelated aspects of integrity and misconduct in proposing, conducting and reporting scientific research:

- What do we know about the occurrence of ethically wrong or questionable behavior in the conduct of research? Misconduct, Dr. Swazey will argue, needs to be defined and dealt with more broadly than the familiar trinity of "falsification, fabrication and plagiarism" contained in federal regulations.
- What are the responsibilities of researchers, their institutions and professional communities seeking to ensure integrity and deal with misconduct, and what are the persisting problems in exercising those responsibilities?

Attendance is required of all Pre- and Post-doctoral trainees, and is strongly recommended for M.D.-Ph.D. and M.A. degree candidates.

Now available for viewing by departments, training directors and trainees:

Ethics and Laboratory Animals

This 40-minute videotape produced by Boston University School of Medicine outlines legal and ethical issues in conducting research using laboratory animals and provides a helpful introduction to issues encountered when use of laboratory animals is contemplated.

Balancing Society's Mandate

Produced by the National Library of Medicine for the National Institutes of Health and the Food and Drug Administration, this 35-minute tape reviews the six criteria used by Institutional Review Boards (IRBs) in considering proposals for research involving human subjects.

Both videotapes are available for short term (1 week) loans from the Dean's office. Please contact Mr. Peter Reich at 638-5240.

BOSTON UNIVERSITY SCHOOL OF MEDICINE
AND
THE DIVISION OF GRADUATE MEDICAL AND DENTAL SCIENCES
PRESENT
THE 1994 SERIES ON

THE RESPONSIBLE CONDUCT OF RESEARCH

A CASE STUDY IN THE ETHICS OF CONDUCTING SCIENCE

PRESENTED IN CONJUNCTION WITH THE SCHOOL OF PUBLIC HEALTH AS A PUBLIC HEALTH FORUM

TUESDAY, MAY 24

4 - 5 PM

ROOM L-112

The reading of this case study will be led by Michael Grodin, M.D., Professor of Philosophy, Medicine, and Public Health; and Director of the Boston University Program in Medical Ethics. A lively discussion is anticipated on how young researchers hurriedly prepare an abstract. Topics include: consequences of the pressure to publish, sharing of privileged information, role and obligation of mentor, and distinctions between honest mistakes and deliberate unethical behavior.

DISCUSSANTS

Theodore Colton, Sc.D.

Professor and Chairman, Department of Biostatistics and Epidemiology, BUSPH

R. Andrew Zoeller, Ph.D.

Assistant Research Professor of Medicine, Assistant Professor of Biophysics

Elizabeth Simons, Ph.D.

Professor of Biochemistry, Research Professor of Biophysics

Leonard H. Glantz, J.D.

Professor of Public Health and Socio-Medical Sciences, and Community Medicine

Bruce Jacobsen

Ph.D. candidate in Pathology/Immunology

Ligaya Stice

M.D.-Ph.D. candidate, Department of Biochemistry/Cancer Research Center

Attendance is required of all Pre- and Post-doctoral trainees, and is strongly recommended for M.D.-Ph.D. and M.A. degree candidates.

BOSTON UNIVERSITY SCHOOL OF MEDICINE
AND
THE DIVISION OF GRADUATE MEDICAL AND DENTAL SCIENCES
PRESENT
THE 1994 SERIES ON

THE RESPONSIBLE CONDUCT OF RESEARCH

STATISTICAL ISSUES IN THE INTEGRITY OF BIOMEDICAL RESEARCH

PRESENTED IN CONJUNCTION WITH THE SCHOOL OF PUBLIC HEALTH AS A PUBLIC HEALTH FORUM

TUESDAY, APR. 26

4 - 5 PM

ROOM L-112

- Choice of a sufficiently large study size
- Guidelines for discarding data
- Hazards of sub-group analysis
- When to stop a study
- Detection of data fabrication
- Abuse and misuse of P-values

PRESENTERS AND DISCUSSANTS

Theodore Colton, Sc.D.

Professor and Chairman, Department of Biostatistics and Epidemiology,
Boston University School of Public Health

Author of Statistics in Medicine (textbook)

Co-Editor, Statistics in Medicine (journal)

Ralph D'Agostino, Ph.D.

Professor of Mathematics/Statistics and Public Health,
Boston University

Expert Consultant in Biostatistics to U.S. Food and Drug Administration,

Director of Statistics and Data Management Unit,
Framingham Heart Study

*Attendance is required of all Pre- and Post-doctoral trainees, and is strongly recommended
for M.D.-Ph.D. and M.A. degree candidates.*

BOSTON UNIVERSITY SCHOOL OF MEDICINE
AND
THE DIVISION OF GRADUATE MEDICAL AND DENTAL SCIENCES
PRESENT
THE 1993 LECTURE AND WORKSHOP SERIES ON

THE RESPONSIBLE CONDUCT OF RESEARCH

SCIENTIFIC INTEGRITY: ETHICS AND VALUES IN THE LABORATORY

Michael Grodin, M.D.

Professor of Philosophy, Medicine, and Public Health

Director, Boston University Program in Medical Ethics

Co-Editor, The Nuremberg Code: Human Rights in Human Experimentation
and Children as Research Subjects: Science, Ethics and Law

Tuesday, Oct. 19

4:30 PM

Baskt Auditorium

THE BOSTON UNIVERSITY POLICY ON MISCONDUCT IN SCHOLARSHIP & RESEARCH

Michael B. Rosen, J.D.

Associate General Counsel, Boston University

Vice-Chairman, Boston University Procedures Committee

Co-Author, Boston University's Policies and Procedures Concerning
Allegations of Misconduct in Scholarship and Research

Tuesday, Nov. 16

4:30 PM

Baskt Auditorium

STATISTICAL ISSUES IN THE CONTEXT OF MEDICAL RESEARCH

Theodore Colton, Sc.D.

Professor of Biostatistics & Epidemiology,

Boston University School of Public Health

Author of Statistics in Medicine (textbook)

Co-Editor, Statistics in Medicine (journal)

Dec. 7

POSTPONED

am

WORKSHOPS ON AUTHORSHIP

Workshops will be held on building bibliographies electronically, scientific writing, and authorship, featuring live, on-line connect time and training in MEDLINE (The on-line version of Cumulated Index Medicus) and SCISEARCH (The on-line version of Science Citation Index). Instructor, Peter Reich, M.P.H., Assistant Professor of Public Health and Director of Special Projects at BUSM. Sign up for workshops at the October 19 lecture. Workshops will be scheduled at convenient times in the student computer lab.

Attendance at a minimum of three of these programs is required of all Pre- and Post-doctoral trainees in basic science or clinical departments, and is strongly recommended for M.D.-Ph.D., Ph.D. and M.A. degree candidates, as well as trainees covered by research grants.

BOSTON UNIVERSITY SCHOOL OF MEDICINE
PRESENTS
THE SECOND ANNUAL SYMPOSIUM
ON
THE RESPONSIBLE CONDUCT
OF RESEARCH

Program

- 8:30 a.m. Welcome - Dean Aram V. Chobanian, M.D.
- 8:40 a.m. Background and Context Herbert H. Wotiz, Ph.D., Professor of Biochemistry, Research Professor of Urology.
- 9:00 a.m. Authorship & Responsibility
- Mary C. Williams, Ph.D., Professor of Medicine and Anatomy & Neurobiology; co-editor, The American Journal of Respiratory Cell and Molecular Biology
- Paul Pilch, Ph.D., Professor of Biochemistry and Research Professor of Biophysics, Editorial Board of The Journal of Biological Chemistry, Biochemica Biologica Acta, and The Journal of Cell Biochemistry
- Richard A. Cohen, M.D., Professor of Medicine and Associate Research Professor of Physiology; Associate Editor, Endothelium; editorial board of other journals
- Dr. Wotiz, Associate Editor, Steroids
- 10:00 a.m. Break
- 10:10 a.m. Federal Policies on Scientific Misconduct
LYLE W. BIVENS, Ph. D.
DIRECTOR, DIVISION OF POLICY, OFFICE OF RESEARCH INTEGRITY U.S. PUBLIC HEALTH SERVICE

THURSDAY • OCTOBER 8, 1992 • BAKST AUDITORIUM • 8:30 A.M.
COFFEE • PASTRIES • JUICE • FRUIT

THE RESPONSIBLE CONDUCT OF RESEARCH

A SYMPOSIUM

FOR THE RESEARCH COMMUNITY AT
BOSTON UNIVERSITY SCHOOL OF MEDICINE

8:30-8:40:	<u>Welcome</u> - Dean Aram V. Chobanian, M.D.
8:40-9:00:	<u>Background and Context</u> . Herbert H. Wotiz, Ph.D., Professor of Biochemistry, Research Professor of Urology, former Director of the Hubert H. Humphrey Cancer Research Center.
9:00-9:30:	<u>The Boston University Policy on Misconduct in Scholarship and Research</u> . Michael Rosen, J.D., Associate Corporation Counsel.
9:30-10:10:	<u>Minimum Standards for Laboratory Record-Keeping</u> . David J. Salant, M.D., Professor of Medicine Nadia A. Rosenthal, Ph.D., Associate Professor of Biochemistry Robert F. Meenan, M.D., Professor of Medicine
10:10-10:30:	<u>Coffee/Juice Break</u>
10:30-10:50:	<u>Outliers: Biostatistics or Ethics</u> . Herbert L. Kayne, Ph.D., Associate Professor of Physiology, Biometrics, Socio-Medical Sciences & Public Health
10:50-11:30:	<u>Ethics and Laboratory Animals</u> . Jerrold Tannenbaum, J.D., Author of <u>Veterinary Ethics</u>
11:30-12:10:	<u>Experimentation with Human Subjects</u> . Leonard Glantz, J.D., Professor of Public Health (Health Law) and Socio-Medical Sciences.

COFFEE & JUICE
PROVIDED!

KEEFER AUDITORIUM
THURSDAY, OCTOBER 3, 1991
8:30 A.M. - 12:30 PM



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PRESENT THE 1999 SERIES ON

the RESPONSIBLE CONDUCT *of* RESEARCH

The 1999 Series

Box Lunch in Hiebert Lounge, 12-1 pm

Thursday, February 18

The Boston University Policy on Scientific Misconduct

Introduction: Norman G. Levinsky, M.D., *Professor of Medicine, Associate Provost*

Policy: Michael B. Rosen, *Associate General Counsel, Boston University*

Tuesday, March 30

High Crimes and Misdemeanors: Collecting and Interpreting Data: Part I

Thomas L. Rothstein, M.D., Ph.D.

Professor, Departments of Medicine and Microbiology; Director, Immunobiology Unit

Thursday, April 15

Collecting and Interpreting Data: Part II

Small group discussions based on problems identified on March 30.

Members of the Committee on the Responsible Conduct of Research (faculty and students/trainees) and guests from Basic Science or Clinical Departments

will meet with small groups of graduate students/trainees to follow up on the March 30th session and to identify subjects and topics for future sessions.

Attendance is required of all Pre- and Post- doctoral trainees and M.D.-Ph.D. and M.A. candidates.



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11. FINAL REPORTS:

Publications and meeting abstracts by the students.

Use of Mammographyh among African-American women, **Yvette Cozier**, MPH,
Julie Palmer, Sc.D, Lynn Rosenberg, Sc.D, Lucile L. Adams-Capbell, Ph.D.
Submitted to Am. J. Public Health, 3/16/99

25-HYDROXYVITAMIN D-1-ALPHA-HYDROXYLASE MRNA EXPRESSION AND METABOLIC ACTIVITY IN CULTURED HUMAN BREAST ADENOCARCINOMA CELLS. **I. Gherson**¹, L.W. Whitlatch², X. Zhu³, X. Kong³, and M.F. Holick⁴. Advisor: M.F. Holick. Departments of Pathology¹, Physiology^{2,4}, Medicine and Dermatology^{3,4}, Section of Nutrition and Diabetes⁴, Vitamin D, Skin and Mineral Research Laboratory^{1,2,3,4}, Boston University School of Medicine.

The enzyme 25-hydroxyvitamin D-1 α -hydroxylase (1 α -OHase) mediates the conversion of the major vitamin D metabolite in the circulation, 25-hydroxyvitamin D₃ (1,25(OH)D₃) to its active form, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). This active form of vitamin D is well known to be a potent inhibitor of cellular proliferation in a number of different epithelial cell types. The 1 α -OHase is a member of the P450 enzyme family. Until recently the 1 α -OHase was thought to be expressed exclusively in the kidney. Our lab has 1) demonstrated the expression of the in extra-renal tissues, i.e., keratinocytes and prostate cells, 2) cloned the extra-renal human 1 α -OHase cDNA and 3) cloned the 1 α -OHase gene. The presence of this enzyme in extra-renal tissues (which also express the vitamin D receptor and are growth-inhibited by 1,25(OH)₂D₃) suggests that it may be involved in an autocrine/paracrine pathway regulating cellular proliferation.

Preliminary mRNA analysis in the MCF-7 breast cancer cell line indicates the expression of the 1 α -OHase in these cells. In addition, metabolic activity studies using normal-phase HPLC examined whether these breast cancer cells could convert 25(OH)D₃ to 1,25(OH)₂D₃ via the 1 α -OHase enzyme. These results suggest that this breast cancer cell line has the enzymatic machinery to synthesize 1,25(OH)₂D₃.

Abstract for Russek Annual Student Achievement Day, Boston University School of Medicine, April 7, 1999

Publications:

1. Trombino AF, RI Near, RA Matulka, S Yang, **LJ Hafer**, PI Toselli, DW Kim, AE Rogers, GE Sonenshein and DH Sherr, "Expression of the Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and the AhR-Regulated *CYP1* Gene Transcripts in a Rat Model of Mammary Tumorigenesis", manuscript submitted for publication February 1999.
2. Rogers AE, LM Sullivan and **LJ Hafer**, "Dietary Fat, Body Weight and Cancer: Contributions of Studies in Rodents to Understanding these Cancer Risk Factors in Humans", manuscript submitted to Toxicological Sciences, 1998.
3. Rogers AE, **LJ Hafer**, YS Iskander and S Yang, "Black Tea and Mammary Gland Carcinogenesis by 7,12-dimethylbenz(a)anthracene in Rats Fed Control or High Fat Diets", *Carcinogenesis*, 19(7): 1269-1273, 1998.
4. Yamaguchi K, RA Matulka, AM Shneider, P Toselli, AF Trombino, S Yang, **LJ Hafer**, KK Mann, X-J Tao, JL Tilly, RI Near and DH Sherr, "Induction of PreB Cell Apoptosis by 7,12-Dimethylbenz[a]anthracene in Long-Term Primary Murine Bone Marrow Culture", *Toxicology and Applied Pharmacology*, 147: 190-203, 1997.

Published Abstracts:

1. Traish A, K Murphy, **L Hafer**, N Savelyeva and A Rogers, "Activation of Human Estrogen Receptor by Tea Extracts", *The FASEB Journal*, Abstract #753, 12(4): A129, 1998.
2. Schmitt H, R Moreland, N Savelyeva, **L Hafer** and A Traish, "*nmt55* Binds to Intracisternal A-Particles Proximal Enhancer Element (IPE) and Polypyrimidine Tract-Splicing Factor (PSF)", *The FASEB Journal*, Abstract #2742, 12(4): A472, 1998.
3. **Hafer LJ**, KE Murphy, AE Rogers, AM Traish and PF Johansen, "Black Tea and Steroid Hormone Receptors in Normal and Neoplastic Mammary Glands in Rats", *Toxicological Sciences*, Abstract #1559, 42(1-S): 317, 1998.
4. Rogers AE, **LJ Hafer**, YS Iskander and S Yang, "Mammary Gland Carcinogenesis, Black Tea and Dietary Fat", *Toxicological Sciences*, Abstract #1558, 42(1-S): 317, 1998.
5. Trombino AF, RA Matulka, S Yang, **LJ Hafer**, AE Rogers and DH Sherr, "Nuclear Expression of the Aryl-Hydrocarbon Receptor (AHR) in 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Tumors and Human Breast Cancer Cell Lines", *Toxicological Sciences*, Abstract #1556, 42(1-S): 316, 1998.
6. Trombino AF, S Yang, **LJ Hafer**, AN Qadri, AE Rogers and DH Sherr, "Modulation of Aromatic Hydrocarbon Receptor Expression in 7,12-Dimethylbenz(a)anthracene-induced Rat Mammary Tumors", *Fundamental and Applied Toxicology*, Abstract#1238, 36(1 pt. 2): 244, 1997.

Abstracts for Poster and Platform Presentations:

1. **Hafer LJ**, AM Traish and AE Rogers, "Estrogen and Progesterone Receptors in Normal and Malignant Mammary Glands in Tea and High Fat Diet-Fed Female Sprague-Dawley Rats", 1999 Henry I Russek Student Achievement Day (Boston, MA), poster to be presented.
2. **Hafer LJ**, KE Murphy, AE Rogers, AM Traish, YS Iskander, S Yang and PF Johansen, "Black Tea, High Dietary Fat and Steroid Hormone Receptors in Mammary Gland Carcinogenesis in Sprague-Dawley Rats", 1998 Henry I Russek Student Achievement Day (Boston, MA), poster presented.
3. **Hafer LJ**, KE Murphy, AE Rogers, AM Traish and PF Johansen, "Black Tea and Steroid Hormone Receptors in Normal and Neoplastic Mammary Glands in Rats", 1998 Society of Toxicology Annual Meeting (Seattle, WA), poster presented.
4. **Hafer LJ**, KE Murphy, AE Rogers and AM Traish, "Black Tea and Estrogen and Progesterone Receptors in the Rat Mammary Gland", 1997 Department of Defense Breast Cancer Research Program Meeting (Washington, DC), platform presentation.
5. **Hafer LJ**, YS Iskander, S Marecki and AE Rogers, "Black Tea and Mammary Carcinogenesis in Rats", 1996 American Association For Cancer Research Workshop -- Histopathobiology of Neoplasia (Keystone, CO), poster presented.

**Expression of the Aryl Hydrocarbon Receptor/Transcription Factor (AhR) and AhR-Regulated
CYP1 Gene Transcripts in a Rat Model of Mammary Tumorigenesis¹**

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Toselli[#], Dong W. Kim[#], Adrienne E. Rogers*, Gail E. Sonenshein[#], and David H. Sherr^{*+}

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Running Title: AhR and CYP1 hyper-expression in rat breast tumors

Key Words: Aryl hydrocarbon receptor, breast tumors, CYP1, tumorigenesis

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07381, Grant DAMD17 94 J4468, Massachusetts Department of Public Health Grant #34088PP1035,
and a Veterans Administration Medical Research Division Center Grant to the Boston Environmental
Hazard Center.

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Schools of Medicine and Public Health, 80 East Concord Street (S-105), Boston, MA 02118.

³Abbreviations: AhR: Aryl hydrocarbon receptor/transcription factor; ARNT: Aryl hydrocarbon receptor
nuclear translocator protein; *CYP1*: genes encoding P450-1 proteins; DMBA: 7,12-
dimethylbenz[*a*]anthracene; IHC: Immunohistochemical; PAH: polycyclic aromatic hydrocarbon(s).

Abstract

It has been suggested that exposure to and bio-accumulation of ubiquitous environmental chemicals, such as polycyclic aromatic hydrocarbons (PAH), contributes to human breast cancer. In animal models, PAH induce tumors in part by activating the aryl hydrocarbon receptor (AhR)/transcription factor. Historically, investigations into the role that the AhR plays in carcinogenesis have focused on its transcriptional regulation of cytochrome P450 (CYP) enzymes which oxidize PAH to mutagenic intermediates and initiate transformation. However, recent studies suggest that the AhR may directly regulate cell growth and/or function. Given the postulated role of the AhR in tumor initiation and growth, it was predicted that: 1) significant levels of AhR would be expressed in tissue predisposed to PAH tumorigenesis, and 2) aberrant AhR and/or AhR-regulated gene expression would accompany malignant transformation. To test these hypotheses, AhR protein and *CYP1* mRNA expression were evaluated in an animal model of breast cancer. Rat mammary tumors were induced by oral gavage with 15-25 mg/kg 7,12-dimethylbenz[*a*]anthracene (DMBA), a prototypic PAH and AhR ligand. AhR-specific immunoblotting and immunohistochemistry demonstrated modest levels of AhR protein localized to myoepithelial cells and, to a lesser extent, ductal epithelial cells in normal rat mammary tissue. In contrast, high levels of AhR were detected in neoplastic mammary tissue comprised predominantly of DMBA-induced fibromas, papillomas, adenomas, or adenocarcinomas. AhR hyper-expression characterized both neoplastic epithelial cells and fibroblasts in the tumor microenvironment. Nuclear AhR localization in neoplastic tissue was suggestive of constitutive AhR activation. *In situ* hybridization studies were consistent with high level *AhR* mRNA expression in the tumor microenvironment and in neoplastic epithelial cells. Quantitative RT-PCR assays indicated a 27.6 fold increase in *AhR* mRNA in neoplastic (2.1 fg *AhR* mRNA/ μ g total RNA) as compared with normal (0.076 fg *AhR* mRNA/ μ g total RNA) rat mammary tissue. While both AhR-regulated *CYP1A1* and *CYP1B1* mRNAs were significantly induced in rat breast tissue within 6 hours of DMBA gavage, only

CYP1B1 mRNA remained elevated in neoplastic tissue 16-18 weeks after DMBA exposure.

Collectively, these results: 1) help explain targeting of breast tissue by carcinogenic PAH, 2) imply that AhR and CYP1B1 hyper-expression represent molecular biomarkers for at least PAH-induced mammary cell transformation, and 3) suggest mechanisms through which the AhR and AhR-regulated gene products may contribute to neoplasia well after exogenous AhR ligands have been eliminated.

Dietary Fat, Body Weight and Cancer: Contributions of Studies in
Rodents to Understanding these Cancer Risk Factors in Humans¹

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Submitted to Toxicological Sciences October, 1998

Dietary Fat, Body Weight and Cancer: Contributions of Studies in Rodents to Understanding These Cancer Risk Factors in Humans: Rogers, A.E., Sullivan, L.M., Hafer, L.J. (1998) *Toxicol. Sci.*

Understanding diet and energy balance as risk factors for breast, colon and other cancers requires information on the contribution of each factor and of interactions among factors to cancer risk. Rodent models for breast cancer provide extensive data on effects of dietary fat and calories, energy balance, body weight gain and physical activity on tumor development. Analyses of the combined data from many studies have shown clearly that quality and quantity of dietary fat and energy balance contribute independently to increased mammary gland tumorigenesis in female rats fed diets high in fat (35-40% of calories) compared to rats fed control diets, approximately 10% of calories as fat (Fay & Freedman, 1997). The methods used permit comparison of experimental and epidemiological data and may be useful in extrapolating between species and developing public health recommendations. In addition to the contributions of lifetime diet composition, intake, energy balance and physical activity to cancer risk, there are questions about the timing and duration of alterations in these factors and about the "dose-response" characteristics of cancer risk to the factors. Endocrine mechanisms may be significant in mammary gland tumor risk, but experimental and epidemiological data indicate that cancers at other sites, such as colon and liver, also are influenced by the factors listed. Other diet and lifestyle factors that influence energy, or specifically fat, metabolism may influence risk for cancers that are promoted by increased intake of fat and calories. Studies of separate and interactive effects of dietary fat, black tea, weight gain and mammary gland tumorigenesis (Rogers, et al, 1998) have been analyzed using adjustment of carcinogenesis end points for body weight; tumor burden and latency were found to be related to weight gain within treatment groups in two of three experiments.

Key words: mammary tumors, calories, dietary fat, weight gain, rats, exercise, black tea

Introduction

The interactions among dietary fat and calories, body mass index, weight gain and physical activity and between those factors and the development of cancer are the subjects of extensive epidemiological and experimental investigation. The results of such investigations are potentially applicable to understanding mechanisms of cancer development and to designing methods for prevention, prognosis and treatment of cancer. Because they are modifiable, the factors listed are of great importance. Of the three major causes of cancer death in the U.S., aside from lung cancer for which the major cause is known, two (breast and colon) are generally thought to be related to dietary fat and calories, body mass, and physical activity; for the third (prostate), data are accumulating that suggest the same conclusions (Huang et al, 1997; Rose, 1997a,b, 1998; Willett, 1998).

In female laboratory rodents the promotion of spontaneous or induced mammary gland carcinogenesis (measured by tumor incidence and latency) by *ad libitum* intake of high fat diets has been demonstrated repeatedly and was, by meta analysis, shown to be independent of total dietary calorie content and intake or final body weight (Freedman, Clifford and Messina, 1990; Fay & Freedman, 1997). In male and female rodents there is considerable evidence for an influence of dietary fat on carcinogen-induced colon tumorigenesis, but enhancement has not been as consistently demonstrable as in the mammary gland (Nauss et al 1987; Reddy et al, 1997). Studies of dietary influences on carcinogenesis in the prostate await development of satisfactory animal models (Rose, 1997, a,b).

In laboratory rodents fed control, generally natural product, diets there is a positive correlation between body weight gain and the incidence of common spontaneous tumors of the mammary gland, liver and pituitary (Seilkop, 1995; Keenan et al 1996; Keenan, , 1998). There is a reduction in spontaneous tumors and also in chemical carcinogenesis in the mammary gland in rats and mice when dietary caloric intake is restricted and weight gain is reduced. In male mice, reduction in spontaneous mammary gland tumors by caloric reduction to 60% of full intake is independent of p53 (Hursting et al, 1997). Feed restriction, leading to 35-50% lower body weight at two years, compared to full-fed controls, reduced spontaneous mammary gland, endocrine and hematopoietic tumors in Fisher 344 (F344) and Sprague-Dawley (S-D) male and female rats (Keenan et al, 1996; Christian et al, 1998). In the most extensively studied mammary tumor model, the 7,12-dimethylbenz(a)anthracene (DMBA)-treated rat, the data indicate that a 10% or greater restriction of feed intake with reduction of body weight yields a detectable decrease in tumorigenesis (Kritchevsky, 1997). However, in full-fed rats an association of tumor incidence or latency with final body weight or weight gain has not been consistently demonstrated. As in epidemiological studies, interactions of the supply and utilization of calories and body mass complicate the interpretation of results.

In a large meta analysis of studies of mammary tumorigenesis in rats and mice fed control or high-fat diets, fat and calorie intake each independently increased tumorigenesis, but there was no clear statistical relationship to final body weight (Freedman et al 1990). Subsequent analyses showed the largest dietary fat effect to be due to N-6-polyunsaturated fats (N-6-PUFA), but saturated fats also were effective (Fay et al 1997; Fay & Freedman, 1997). Using the same model, the authors examined epidemiological data across countries and reported evidence for similar but less marked effects of dietary fats (Fay & Freedman, 1997). They then applied the model to hypothetical diet designs and calculated odds ratios for breast cancer. They reported that, in this model as in the animal studies, fat calories (except from fish oil) would have a greater effect than nonfat calories, i.e. that the fat effect on carcinogenesis in the breast is not purely a calorie effect, and that N-6-PUFAs have the greatest effect.

A recent review of studies of the influence of physical activity or exercise on mammary gland carcinogenesis in rats concluded that there is consistent evidence that intensive exercise (> 70% maximal aerobic capacity or exercise to exhaustion) reduces carcinogenesis endpoints by more than 50%; studies of less intensive exercise did not yield evidence of a consistent

effect (Thompson, 1997). In that review Thompson (1997) discusses important points about methods to study exercise in rodents, definitions of terms and major questions to be answered about the influence of exercise type, intensity and duration on carcinogenesis and the mechanisms by which exercise might act. In additional studies in rats of the effects of caloric balance on mammary gland tumorigenesis, using either exercise to increase energy expenditure or diet restriction to reduce intake, it has been reported that adrenal corticosteroid responses to exercise or diet restriction rather than caloric balance per se are responsible for reducing tumorigenesis (Gillette et al, 1997; Zhu et al, 1997). Harris et al (1995) studied DMBA-induced mammary gland tumorigenesis in full-fed S-D rats, rats restricted to 60% of the energy intake of full-fed and rats fed in cycles of 2 days of feeding at the level of the full-fed rats and 2 days of 60% feeding. The cycled rats had 81% of the energy consumption of the full-fed rats, weighed 15% less and had a slightly but not significantly lower tumor incidence and burden. The continuously restricted rats weighed 28% less than the full-fed rats and had significant reductions in tumor endpoints. Both restricted groups had elevated serum corticosterone and reduced estradiol, so the significance for carcinogenesis of the corticosterone changes with feed restriction is not clear.

We have carried out three experiments examining DMBA-induced mammary tumorigenesis in female S-D rats fed control or, in one experiment a high-fat, diet and given black tea to drink. We found no consistent effect of tea on tumorigenesis in rats fed control diet, but we did find the expected increase in rats fed the high-fat diet and no statistical increase in tumorigenesis in rats fed the high-fat diet and given tea compared to rats given water (Rogers et al, 1998). We have examined the data on individual body weight gain and tumorigenesis end points from these three experiments to determine if there was a relationship between weight gain and one or more tumorigenesis end points (tumor number, weight, burden and latency). In addition we were interested to determine if tea, which has the potential to reduce weight gain, or dietary fat, which has the potential to increase weight gain, would change any relationship we might detect between tumorigenesis and body weight gain.

Methods

Three experiments were performed in female S-D rats, comparing tumorigenesis in: (Experiment 1), rats given by gavage 25 mg/kg DMBA, fed AIN-76A diet and given 1.25% or 2.5% black tea extract or water to drink; (Experiment 2), rats given 15 mg/kg DMBA, and the same diet and drinking fluids as in Experiment 1; (Experiment 3), rats fed AIN-76A or a high-N-6-PUFA diet and given 15 mg/kg DMBA and 2% tea or water to drink. The rats readily accepted tea and ate and gained weight normally. Total tea intake in Expt. 1 represented 58 ± 13 or 106 ± 22 grams of extracted tea leaf (Rogers et al, 1998).

Analysis of variance was used to compare unadjusted (for weight) end points between comparison groups. Analysis of covariance was used to compare end points adjusted for weight gain over 16 weeks. To understand the adjustment, the relationships between weight gain over 16 weeks and each end point within each comparison group were estimated using simple linear regression analysis. The difference between groups in the relationships between weight gain and each end point was analyzed by testing whether the interaction between weight gain and group assignment was statistically significant. A p-value ≤ 0.01 was considered statistically significant. All analyses were performed using SAS 6.12. Sample sizes of at least 10 rats per independent variable are required for these analyses.

Results

Relationships between body weight gain and tumorigenesis end points over the entire experiment were similar in all groups irrespective of tea or diet (Table 1). Tumor number and burden at termination increased significantly with the amount of body weight gain over the 16 weeks that rats were studied, beginning at 4 weeks of age. Weight gains during any four-week

period of observation (from entry to 12 weeks after administration of DMBA at 8 weeks of age) appeared to be equally effective.

In Experiment 1, considering all DMBA-treated rats together, the number of tumors per rat at termination of the study increased by 1.65 per 100 grams body weight gain between week 4 and week 16. Tumor burden per tumor-bearing rat increased by 3.16 g per 100 grams body weight gain over the same period, and mean tumor weight increased by 0.68 g. Tumor latency (first tumor) decreased by 8.32 days per 100 grams body weight gain, and latency to second tumor by 2.49 days (Table 1).

In Experiments 2 & 3 a smaller dose of DMBA was administered; tumorigenesis was slower, and the numbers were smaller. In Experiment 2 there were no important changes in end points with weight gain. In Experiment 3 the responses of tumor end points to weight gain were generally in the same direction as in Experiment 1; in separate examinations of the groups the responses to weight gain were increased by the high fat diet in water-drinking rats but not in tea-drinking rats (Table 1). The individual groups are too small to permit further statistical analysis.

Adjustment of the tumor data for body weight did not alter the conclusions already reported: significant increases in tumor number, tumor burden and mean tumor weight and decreases in latency in rats fed the high-fat diet compared to rats fed control diet, and, in rats fed the high-fat diet and given tea, no significant changes in tumorigenesis end points compared to tea-or water-drinking rats fed control diet (Rogers *et al*, 1998). Neither tea nor the high fat diet consistently altered rats' weight gain in the 3 experiments.

In the analysis of Freedman *et al* (1990) of effects of dietary fat on mammary tumorigenesis, final body weight was found to increase about 1% for 10% added dietary fat calories in S-D rats but was not statistically related to the increase in tumor incidence with increased dietary fat. However those authors did not examine the individual tumor end points and weight gain data as was done in the present study.

Discussion

In the 3 experiments it is clear that there is an effect of body weight gain on tumorigenesis in this model and that the magnitude of the effect varies among experiments. Adjustment of the results for weight gain is appropriate and can make an appreciable difference in the group means for the end points; it did not, however, change the final outcomes previously reported. In experiments in which there are larger intergroup differences in weight gain, the adjustments may make a significant difference.

The effects of tea, coffee and caffeine on cancer risk have been of interest and could be related to body weight. In the experiments discussed here the rats drank an average of approximately 10-30 mg/kg/day of caffeine, comparable to the highest levels of consumption reported in Western countries (7-15 mg/kg/day in Denmark, Barone & Roberts, 1996). There are many reports of a protective effect of tea against tumorigenesis in several animal models. Weisburger *et al* (1997) reported protection by tea in DMBA-treated rats fed a high fat diet; inconsistent effects of coffee have been reported in rats and mice (Welsch *et al* 1988; Welsch, 1994). In the gastrointestinal and respiratory tract tumor models in which tea is a chemopreventive agent, decaffeinated teas have generally been active although somewhat less so than full tea. Recently a significant chemopreventive effect of 2% black tea and of caffeine alone was demonstrated in the male 344 rat lung tumor model given 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone (NNK, Chung *et al* 1998). In that study caffeine and tea did not significantly reduce the rats' body weights.

Intake of relatively high levels of coffee has been reported related to reduced risk for cutaneous malignant melanoma in women (Veierod *et al* 1997) and for carcinoma of the colon in men and women. (Favero *et al*, 1998), but the epidemiological literature on coffee and tea and cancer risk is highly inconsistent (Yang *et al*, 1996; Kohlmeier *et al*, 1997). Coffee intake had no effect on breast cancer risk in a recent study (Tavani *et al* 1998).

A major question that has not yet been adequately addressed in animal models is the role of physical activity in body weight and in carcinogenesis. Thompson's (1997) review and discussion of results and methodological problems is valuable. Shephard (1996) and Shephard and Futcher (1997) presented detailed tables and analyses of studies of relationships between physical activity or exercise and cancer in laboratory animals and people that were reported between 1980-1997. They concluded that, in a total of 25 reports of studies in animals, there were 15 that showed positive findings of reduction of chemical carcinogenesis in exercised animals, 7 in which exercise may have been effective under certain conditions and 3 in which exercise did not reduce carcinogenesis. Effects of the type and intensity of exercise and of diet, calorie balance and body weight could not be determined from the studies reviewed.

In the epidemiological studies reviewed, risk ratios were inversely related to physical activity-exercise indicators in men for all cancers combined, colorectal adenomas, and malignant colon, prostate, testicular and, possibly, lung tumors. In women the inverse relationship was found for colon, premenopausal breast and, perhaps, uterine tumors. The authors discuss issues of assessment, timing, type and intensity of physical activity, baseline physical condition, body mass and other variables. The studies reviewed indicate a potential reduction overall in cancer incidence by about 46% by increasing physical activity (Shephard, 1996; Shephard and Futcher, 1997). In a brief discussion of potential factors or mechanisms related to the reduction of cancer risk by physical activity, Shepherd (1996) lists diet and other life-style qualities, gastrointestinal tract transit time, free radicals, body type (genetic or acquired), energy balance, weight gain (time and amount) and endocrine responses.

In another recent review, Oliveria & Christos (1997) add consideration of immunological factors in general and of prostaglandins F_2 alpha and E_2 specifically for colon cancer in evaluating mechanisms of an exercise effect. Conclusions from a recent large prospective study of breast cancer risk (Thune et al, 1997) were that physical activity was inversely related to breast cancer risk. The risk reduction was most marked in premenopausal women, women less than 45 years old at entry into the study and in lean women (body mass index <22.8). The lowest risk, 0.28, 95% CI, 0.11-0.70, was lean women who exercised at least 4 hours per week; the association was shown for both pre- and postmenopausal women. Body mass index and fat or energy intake did not influence risk. Women active in leisure time had only slightly greater energy intake than sedentary women and were, consequently, leaner and had lower net available energy. The authors raise the possibility of genetic determination of physical activity as well as of body type and breast cancer risk. They propose also that the lower serum triglycerides in active women permit greater binding of estradiol to sex hormone-binding globulin with reduction of tissue exposure to estradiol. Mezzetti et al (1998) reported in a different population that relatively low body mass (<23.3) and high physical activity were associated with reduced breast cancer risk in postmenopausal women.

In a detailed review, analysis and discussion of recent studies (Gammon et al (1998) conclude that, while there are many studies reporting that physical activity reduces breast cancer risk, consistent evidence on the importance of timing, intensity and frequency of physical activity is lacking, and methods for determining these variables are inadequate. They propose that methods to characterize physical activity be improved and validated to permit evaluation of lifetime physical activity and to examine interactions of activity, diet and weight.

The large literature on colon cancer risk and physical activity can be criticized similarly. Slattery et al (1997) reported a study in which they addressed the questions of interactions of lifetime physical activity, energy balance, body mass index and colon cancer risk. They found increased risk with the lowest level of lifetime vigorous leisure-time activity, high energy intake and large body mass index. If all three risk factors were present the odds ratio was 3.35, 95% CI, 2.09-5.35. High physical activity reduced significantly the effect of the other two factors. The magnitude of the effects of body mass index and energy intake decreased with age.

Studies of interactions of physical activity, diet and weight in determining cardiovascular disease risk may be of help. Since low fat diets designed to reduce LDL cholesterol and coronary heart disease risk may reduce also HDL cholesterol unless weight loss or increased physical activity or both are also present (Wood et al, 1988, 1991, Stefanik et al, 1998; Expert Panel, 1993), research results in this area may be highly relevant to questions posed in cancer research.

In summary, in epidemiological and laboratory animal studies the influences of diet, body weight and physical activity on cancer risk are being elucidated. Definitive studies require better methods and more specific questions. If studies in animals can be designed or combined to provide larger groups, certain statistical methods used in epidemiological investigations may be applicable, such as the adjustment for body weight gain described herein.

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Black tea and mammary gland carcinogenesis by 7,12-dimethylbenz(a)anthracene in rats fed control or high fat diets

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Epidemiological studies suggest that tea may reduce cancer risk, and in laboratory rodents, chemopreventive effects of tea or purified extracts of tea have been demonstrated in lung, gastrointestinal tract and skin. There is some evidence of chemoprevention by tea in the mammary gland, but the data are not conclusive. In order to evaluate more fully the possible influence of black tea on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary gland tumors in the female S-D (Sprague-Dawley) rat, three large studies were performed: experiment 1, tumorigenesis in rats fed AIN-76A diet and given 25 mg/kg DMBA and 1.25 or 2.5% whole tea extract or water to drink; experiment 2, tumorigenesis in rats given 15 mg/kg DMBA and the same diet and fluids as in experiment 1; experiment 3, tumorigenesis in rats fed control or HF (high fat, corn oil) diet and given 15 mg/kg DMBA and 2% tea or water to drink. Tea was given throughout the experiment; DMBA was given by gastric gavage at 8 weeks of age. There was no consistent effect of tea on tumorigenesis in rats fed AIN-76A diet; there was, however, evidence in experiment 3 of a reduction of tumorigenesis by tea in rats fed the HF diet. In experiment 3, rats fed the HF diet and given water showed the expected increase in tumor burden (number and weight) compared with rats fed control diet. However, rats fed the HF diet and given 2% tea showed no increase in tumor burden; their tumor burden was significantly lower than in rats fed the HF diet and given water ($p < 0.01$) and was not different from rats fed control diet and given water or tea. In addition, in experiment 3, the number of malignant tumors per tumor-bearing rat was increased by the HF diet in water-drinking rats ($p < 0.01$) but not in tea-drinking rats. Therefore, it appears that tea partially blocked the promotion of DMBA-induced mammary tumorigenesis by the HF diet.

Introduction

Epidemiological studies strongly suggest that diet components are responsible in part for geographic and cultural differences in cancer site and incidence. For example, fruits and vegetables, soybeans, grains and tea are thought to contain nutrient and non-nutrient substances that reduce cancer risk in the breast, prostate and gastrointestinal tract; in contrast, fats are postulated to increase cancer risk at the same and other sites (1-5). These and other diet components have been evaluated in laboratory animal tumor models (1,5-8). The diet components considered here in relation to breast cancer, namely black tea and fats high in N-6-polyunsaturated fatty acids (N-6-PUFA), are con-

sumed in large amounts by people in many parts of the world and may influence cancer risk.

Recent publications of epidemiological studies indicate a possible reduction of esophageal, rectal, pancreatic and colon cancer risk associated with green tea consumption in China; studies in western populations have not yielded consistent results on effects of black tea on cancer risk at any tissue site (9-16). Epidemiological data on tea and cancer risk have been extensively reviewed recently by Yang *et al.* (9) who concluded that the data are suggestive of cancer risk reduction by tea at some sites but not consistently. Kohlmeir *et al.* (11) concluded that there may be some protection by tea in high cancer risk groups, but the evidence is weak.

In laboratory rodents, extracts of green or black tea given in place of drinking water or added to feed reduce carcinogenesis by certain nitrosamine and polycyclic aromatic hydrocarbon carcinogens in the lung, gastrointestinal tract, liver and skin, and by UV light in the skin (9-10,17-21). Whole aqueous extracts, decaffeinated extracts and purified components of tea have been found effective to varying degrees in the different tumor models. The most extensively studied preparations reported have been aqueous extracts of green tea and its major polyphenol, (-)-epigallocatechin-3-gallate (EGCG), both of which have chemopreventive activity in the organs listed. Mechanisms postulated for the anti-carcinogenic effects of tea extracts include antioxidant activity and alteration of xenobiotic metabolism (9-10).

The epidemiological evidence for an effect of total dietary fat intake on breast cancer risk is not consistent, but a recent review (2) concluded that the weight of evidence is that postmenopausal breast cancer risk is associated with increased dietary fat intake. Attempts to detect age, endocrinological and body size characteristics as well as tumor characteristics that might clarify a relationship between dietary fat and breast cancer risk or mortality have been suggestive of increased risk of mortality with higher fat intakes (22,23) or have yielded negative results (24,25). Further studies of interactions among menopausal status, body mass and serum hormones (26,27) may contribute to clarification of the effects of dietary fat on breast cancer risk.

Diets high in N-6-PUFA are consistent and relatively powerful promoters of mammary gland tumorigenesis in laboratory rodents. Other types of fat also may be promoters. N-6-PUFA and other fats may enhance initiation of tumors as well as promotion, but their greatest and most consistent effects are as promoters (1,7,8,28).

Effects of tea have not been evaluated fully in rodent breast cancer models. The experiment that have been reported generally have yielded data that suggest protection by tea or its components but have not shown statistically significant effects. Hirose *et al.* (29) reported that female Sprague-Dawley (S-D) rats given 50 mg/kg DMBA at 7 weeks of age and, beginning one week later, fed a natural product diet containing 1% green tea catechins (of which 53.9% was EGCG) survived

longer and showed somewhat increased tumor latency and decreased tumor size compared with controls not fed tea. They reported a similar result in female F344 rats fed 2% 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) plus 1% green tea catechins in a natural product diet (30). Weisburger *et al.* (31) reported that female S-D rats fed a high N-6-PUFA (23.5% corn oil) diet that promotes mammary tumorigenesis, given 1.25% black tea extract to drink from 6 weeks of age, and given 5 mg DMBA (equivalent to ~25 mg/kg) by gavage at 7 weeks of age, had fewer mammary fibroadenomas than rats given water; both groups had similar numbers of adenocarcinomas. In the same laboratory in the 2-amino-3-methylimidazo-[4,5-f] quinoline (IQ) mammary tumorigenesis model, tea-drinking rats developed more tumors than controls, but the tumors were smaller and had longer latencies than in controls. Consumption of 1% black tea in a purified diet for 2 weeks before DMBA exposure reduced DMBA-DNA adducts in the mammary glands of S-D rats (32), which would predict an effect of tea on initiation of carcinogenesis.

Fujiki *et al.* (33) found no effect of 0.1% EGCG in drinking water on murine mammary tumor virus (MMTV) tumorigenesis in SHN mice; Sakata *et al.* (34) found no effect of green tea extract (0.1 and 0.05%) in drinking water in the same model.

Liao *et al.* (35) reported that i.p. daily injection of EGCG (1 mg) inhibited growth of tumors from human breast cancer MCF-7 cells implanted subcutaneously in BALB/c female nude mice that carried 17- β estradiol implants. Komori *et al.* (36) reported that green tea catechins (EGCG 85%) and whole green tea extract inhibited growth of two human breast cancer cell lines (MCF-7 and BT20) in culture.

In summary, there is suggestive evidence that green tea catechins or whole green or black tea extracts reduce DMBA- or PHIP-induced mammary tumorigenesis in female rats and that similar preparations inhibit growth of human breast cancer cell lines transplanted into mice or cultured *in vitro*. Similar tea preparations did not reduce tumorigenesis by IQ in rats or by MMTV in SHN mice.

In order to evaluate more fully the possible influence of black tea on DMBA-induced mammary gland tumors in the female S-D rat, three large studies were performed: experiment 1, comparison of tumorigenesis in rats fed AIN-76A diet and given 25 mg/kg DMBA and 1.25 or 2.5% tea extract or water to drink; experiment 2, comparison of tumorigenesis in rats given 15 mg/kg DMBA and the same diet and drinking fluids as in experiment 1; and experiment 3, comparison of tumorigenesis in rats fed control or high fat (HF) diet, and given 15 mg/kg DMBA and either 2% tea or water to drink.

Materials and Methods

Female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), 4–5 weeks of age, were housed individually in environmentally controlled animal quarters and handled according to the NIH guidelines. They were fed AIN-76A (37,38) diet throughout the experiment (experiments 1 and 2) or fed AIN-76A diet before DMBA exposure and then, 96 h later, divided into groups and fed either AIN-76A diet, or an HF diet that was nutritionally equivalent on a caloric basis to the AIN-76A diet (experiment 3). The HF diet contained 24% vitamin-free casein, 24% corn oil, 30.8% sucrose, 9.3% cornstarch, 1.2% AIN vitamin mix, 4.2% AIN mineral mix, 0.24% choline bitartrate, 0.36% DL-methionine and 5.9% α -cellulose.

Black tea (World Blend Tea, Southern Tea Co., Marietta,

Table 1. Treatment groups

Experiment	Group	No. of rats	Tea	Water	DMBA ^a
1 and 2 ^b	C1	10	0	+	0
	C2	40	0	+	+
	C3	40	0	+	+
	T1	10	2.5%	0	0
	T2	40	1.25%	0	+
	T3	40	2.5%	0	+
3 ^c	C1	10	0	+	0
	C4	20	0	+	+
	CF4	30	0	+	+
	T1	10	2%	0	0
	T4	20	2%	0	+
	TF4	30	2%	0	+

^aExperiment 1, 25 mg/kg in 0.2 ml sesame oil by gastric gavage at 8 weeks of age; experiments 2 and 3, 15 mg/kg in 0.2 ml sesame oil by gastric gavage at 8 weeks of age.

^bAll rats fed AIN-76A diet throughout with free access except C3 rats; they were matched and individually pair-fed to T3 rats.

^cAll rats were fed AIN-76A diet before and until 96 h after DMBA administration; from that time until termination of the experiment, CF4 and TF4 rats were fed the high N-6-PUFA diet. All rats had free access to feed.

GA) was formulated and supplied under the auspices of the Tea Trade Health Research Association. The tea was a mixture of leaves grown and processed in the major tea growing countries; the percent composition from each source was constant in all lots. A different lot was used in each of the three experiments; only one lot was used for an experiment. The tea (2.5% in experiments 1 and 2, 2% in experiment 3) was brewed three times per week in a Bunn® automatic basket tea maker using deionized water and was supplied fresh in calibrated bottles to the rats at that concentration or diluted to 1.25% (experiments 1 and 2). Two days later the remaining tea was measured, and fresh tea was given. Controls were given water from the same deionizing system on the same schedule. The rats were introduced to tea in increasing concentrations over a two-week period beginning at their entry into the laboratory in experiment 1. This proved unnecessary and was not done in subsequent experiments.

The groups are summarized in Table 1. In experiments 1 and 2, there were four DMBA-treated groups: two drinking water (C2 and C3) and two drinking tea (T2 and T3). Group T2 rats drank 1.25% tea, and they and C2 rats were given unlimited access to feed. Group T3 rats drank 2.5% tea and had unlimited access to feed; group C3 rats were individually matched by weight to T3 rats and pair-fed three times/week to the matched rat throughout the experiment. There were, in addition, two groups, 10 rats each, not given DMBA and given water (C1) or 2.5% tea (T1) and free access to feed. In experiment 3 there were four DMBA-treated groups, two fed AIN-76A diet and given either 2.0% tea (T4) or water (C4) and two fed the HF diet and given either 2.0% tea (TF4) or water (CF4).

In all experiments, the rats were weighed weekly. DMBA, (25 mg/kg in experiment 1, or 15 mg/kg in experiments 2 and 3) in 0.2 ml sesame oil, was administered by gastric gavage in a single dose to rats 8 weeks of age. Beginning 4 weeks later, rats were palpated weekly for tumor. Rats were killed and necropsied when they bore tumors that were 3–4 cm in diameter or were ulcerated; all rats remaining were killed by CO₂ inhalation and necropsied 16–18 weeks after DMBA administration.

All mammary glands and tumors were rapidly excised; tumors were weighed and sectioned; sections were fixed in 10% neutral buffered formalin (experiment 1) or 4% paraformaldehyde (experiments 2 and 3) or frozen on dry ice and held at -80°C for histochemical, biochemical and molecular studies (to be reported separately). Mammary glands were similarly fixed or frozen. Fixed tissues were processed, embedded, cut and stained with hematoxylin and eosin using routine methods.

Statistical analysis of results was performed using the programs SPSS 7.0 and SAS. The cumulative probability of bearing a palpable tumor over time was analyzed by Wilcoxon and Log-Rank tests. Chi-squared statistics were calculated to compare tumor incidences, among groups. ANOVA statistics with appropriate post-hoc tests (Scheffe's and Tukey's B) were used to analyze and compare body weight, tumor number and tumor weight by treatment group and by pathology.

Results

The rats readily accepted tea as their fluid source and ate and gained weight normally. In experiments 1 and 2, neither concentration of tea was associated with a statistical change in weight gain (data not shown). In experiment 3, rats fed the HF diet (TF4 and CF4) had identical weight gains that were slightly, but not statistically, greater than water-drinking rats fed the control AIN-76A diet (C4); tea-drinking rats fed the AIN-76A diet gained weight normally until 15 weeks of age but then showed reduced weight gain and weighed about 10% less than the C4 rats at termination of the experiment (data not shown).

Fluid intake was highly variable; in experiment 1 the tea-fed rats' daily average intake increased from 30 ml at 10 weeks of age to 38 ml at 16 weeks of age. Total tea intake over the entire experiment represented 58 ± 13 (T2) or 106 ± 22 (T3) g extracted tea leaf. The C2 and C3 groups had identical water intakes that increased on average from 41 ml at 10 weeks to 45 ml at 16 weeks of age. The fluid intakes in experiment 2 were similar in all respects to the intakes in experiment 1; intake was not measured in experiment 3.

In the three experiments, there was no consistent effect of tea on tumorigenesis in rats fed the AIN-76A diet; there was some evidence of a reduction of tumorigenesis by tea in experiment 3 in rats fed the HF diet.

Cumulative probability of tumor in experiment 1 was higher in the T2 group than in the C2 group ($p = 0.04$) and was greater, but not statistically so, in C3 than in T3 (Figure 1). In experiment 2, cumulative probabilities of tumor in both water control groups (C2 and C3) were higher than the corresponding tea-drinking groups (T2 and T3); there were no statistically significant differences. The lower dose of DMBA in experiment 2 induced a lower cumulative probability of tumor in all groups compared with experiment 1 (Figure 2).

In experiment 3, rats fed the HF diet showed the expected increase in cumulative probability of tumor compared with rats fed the control AIN-76A diet (C4 versus CF4, $p = 0.003$; T4 versus TF4, $p = 0.05$). Tea did not statistically have an effect on the cumulative probability of tumor, although TF4 was somewhat lower than CF4 (Figure 3).

In experiments 1 and 2, tumor incidence, number and weight did not differ consistently or statistically between tea-fed and water-fed rats (Table 2). However, in rats fed the HF diet and given 2% tea (TF4) in experiment 3, tumor burden and total

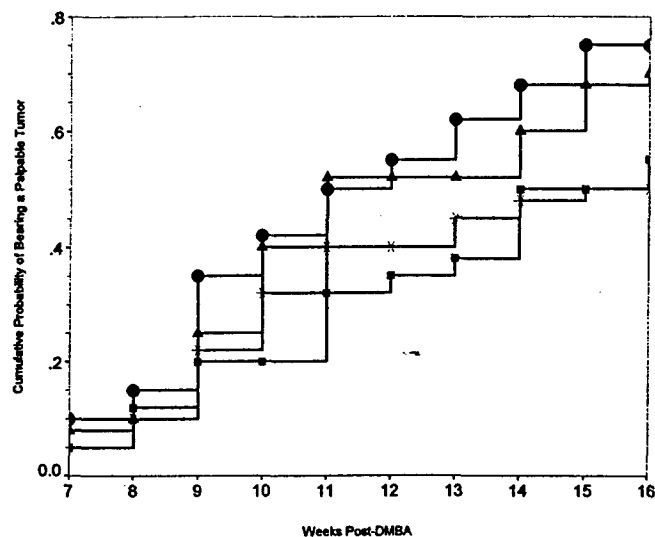


Fig. 1. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 1. ■, C2; ▲, C3; ●, T2; *, T3.

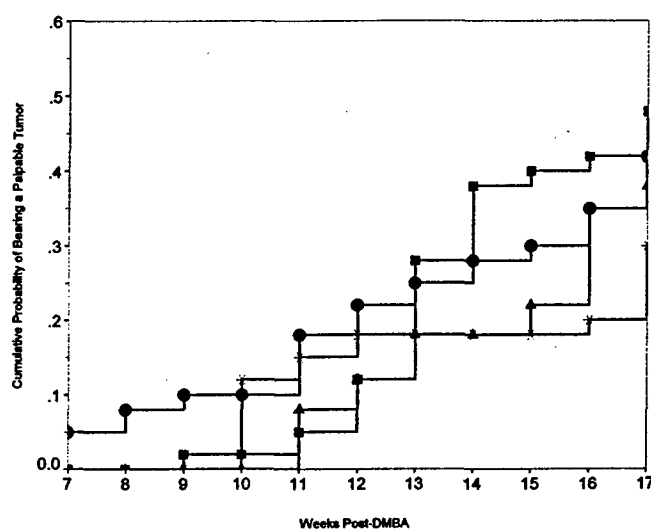


Fig. 2. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 2. ■, C2; ▲, C3; ●, T2; *, T3.

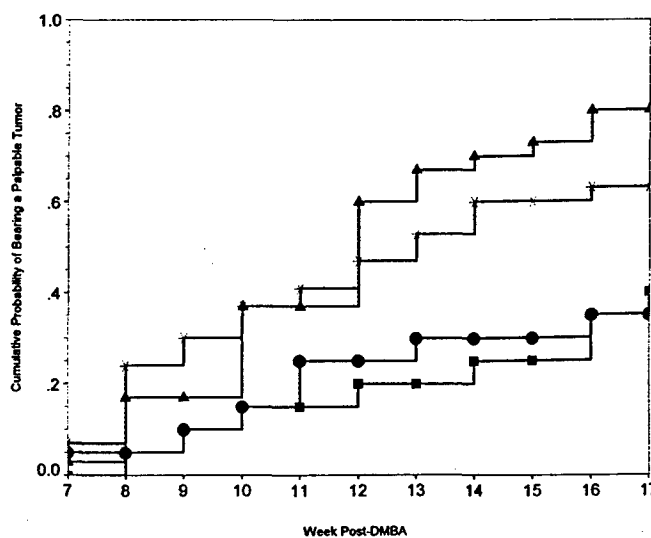


Fig. 3. Cumulative probability of bearing a mammary tumor in DMBA-treated rats in experiment 3. ■, C4; ▲, CF4; ●, T4; *, TF4.

Table 2. DMBA-induced mammary tumor incidence, number and burden in female S-D rats drinking tea or water and fed control or high N-6-PUFA diets

Group	% tumor incidence ^a	No. of tumors ^{a,b} (per tumor-bearing rat)	Total tumor weight (g) ^b (per tumor-bearing rat)
Experiment 1 ^c			
C2	60 (58)	2.9 ± 1.7 (2.4 ± 1.4)	4.0 ± 4.5
T2	75 (75)	3.4 ± 2.3 (3.0 ± 2.1)	5.6 ± 7.5
C3	62 (62)	2.8 ± 1.9 (2.2 ± 1.4)	4.7 ± 6.3
T3	68 (62)	3.2 ± 2.3 (2.7 ± 2.0)	5.4 ± 5.9
Experiment 2 ^c			
C2	62 (50)	2.0 ± 1.2 (1.4 ± 1.1)	3.0 ± 3.4
T2	40 (22)	1.9 ± 1.2 (1.1 ± 1.2)	3.1 ± 4.1
C3	42 (28)	1.7 ± 0.8 (1.0 ± 1.0)	1.7 ± 3.1
T3	40 (32)	2.1 ± 1.8 (1.2 ± 1.1)	2.7 ± 3.1
Experiment 3 ^d			
C4	70 (45)	1.6 ± 0.9 (1.0 ± 1.0)	1.0 ± 0.9
T4	35 (20)	2.0 ± 1.8 (1.3 ± 1.8)	1.9 ± 1.4
CF4	80 (70)	4.1 ± 2.6 ^e (3.1 ± 2.2)	6.0 ± 4.1 ^f
TF4	67 (50)	2.6 ± 1.5 (1.9 ± 1.6)	2.9 ± 2.8

^aMalignant tumor incidence and number in parentheses.^bMean ± standard deviation.^cForty rats per group; C2, water; T2, 1.25% tea; C3, water, pair-fed to T3; T3, 2.5% tea.^dC4 and T4, 20 rats each; CF4 and TF4, 30 rats each; C4, water; T4, 2% tea; CF4, high fat diet + water; TF4, high fat diet + 2% tea.^eSignificantly greater than C4 ($p < 0.01$) for all tumors and for malignant tumors.^fSignificantly greater than C4 ($p = 0.001$) and than TF4 ($p = 0.01$).

tumor weight per tumor-bearing rat, was statistically reduced ($p < 0.01$) compared with rats fed the HF diet and given water (CF4). Tumor number also was reduced in TF4 rats compared with CF4 rats, but the reduction was not significant (Table 2).

In experiment 1, 85% of tumors were malignant, 62% were malignant were malignant in experiment 2, and 76% were malignant were malignant in experiment 3. In experiments 1 and 2, there was no effect of tea on the incidence of benign or malignant tumors. In experiment 3, the number of tumors and of malignant tumors per tumor-bearing rat was increased by the HF diet in water-drinking rats (C4 versus CF4, $p < 0.01$) but not in tea-drinking rats which failed to show the increase associated with the HF diet. Therefore, tea partially blocked the promotion of tumorigenesis by the HF diet.

Discussion

In the three bioassay experiment, the model responded as expected to DMBA at the two doses given and to the amount of corn oil in the diet (7–8). The ingestion of black tea had no consistent effect on mammary gland carcinogenesis in rats fed the AIN-76A diet, but tea did reduce tumorigenesis somewhat in rats fed the HF diet. In this group (TF4, experiment 3) tea ingestion significantly reduced tumor burden compared with rats drinking water. The group had also a somewhat reduced cumulative probability of bearing a tumor, but the reduction was not statistically significant. The effects of tea on DMBA tumorigenesis in rats reported previously in other laboratories have also been detected as a reduction of tumor multiplicity or size, and the Weisburger *et al.* report (31) of reduced DMBA tumor multiplicity in black-tea-drinking rats was obtained in rats fed a high corn oil diet. Therefore, it appears that black tea may reduce DMBA mammary tumori-

genesis in rats, particularly if they are eating a diet high in N-6-PUFA.

Since the rats were ingesting tea before and after DMBA exposure, an effect on initiation, promotion or progression could have been detected in any of the experiments. The results of experiment 3 suggest that tea may reduce the well-known promoting effect of N-6 PUFA on mammary gland tumorigenesis (1,7,8,28). The significance of this effect has been re-emphasized by a recent updated meta analysis of data from a large number of studies in rats and mice (39). The mechanism by which N-6 PUFA promotes mammary tumorigenesis is unknown, despite many investigations of endocrine-related and other hypotheses (1,28,40). Further studies in this model might be enhanced by exploration of interactions of N-6-PUFA with tea.

Black tea intake, characterized in a large cohort of people in the Netherlands, was, at the highest level established of ≥ 5 cups/day, 525 ml or ~ 0.03 ml/cm²/day of 1% tea (41). The rats ingested an average of 0.1 ml/cm²/day of 1.25 or 2.5% tea. Therefore, one can conclude that adequate and not grossly excessive levels of intake were tested.

Mammary gland carcinogenesis is highly sensitive to dietary energy supply as well as to specific fats. The concentration of tea (2.0%) used in experiment 3 had no significant effect on body weight in rats fed the HF diet (TF4) compared with controls (CF4); therefore, the reduction in tumor number in TF4 rats is not attributable to a change in weight gain.

Whole coffee extract and caffeine, an alkaloid component of tea and coffee, have been studied in the S-D rat DMBA tumorigenesis model. The results vary somewhat with timing and amount of coffee or caffeine ingestion, but they generally indicate reduction of tumor multiplicity and no change in incidence or latency when coffee or caffeine is given at initiation and no effect on promotion (42,43). Since caffeine may be present in polyphenol extracts of tea (21), it could contribute to the reported effects of tea and its extracts.

Interactions of tea or tea extracts with the estrogen receptor (ER) in the mammary glands of rats in the experiment reported here and with the ER of calf uterus *in vitro* have been found (44). Such effects have been suggested by other studies (36).

Further studies in this model, using both tea preparations and high N-6-PUFA diets, should yield information useful in chemoprevention and in understanding the basic mechanisms in mammary gland tumorigenesis.

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Induction of PreB Cell Apoptosis by 7,12-Dimethylbenz[a]anthracene in Long-Term Primary Murine Bone Marrow Cultures¹

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Numerous studies demonstrate that polycyclic aromatic hydrocarbons (PAH) suppress immunity by modifying the function of both B and T cells. Relatively few studies have assessed the effects of these common environmental chemicals on immature lymphocytes. In the present study, long-term primary bone marrow cultures were employed to investigate the effects of a prototypic PAH and aryl hydrocarbon receptor (AhR) agonist, 7,12-dimethylbenz[a]anthracene (DMBA), on immature B lymphocytes. In this system, immature preB cells are maintained in a supportive microenvironment provided by bone marrow stromal cells. Results presented here demonstrate that (1) exposure of primary bone marrow cultures to DMBA results in preB cell death by apoptosis; (2) notably low doses of DMBA ($\geq 10^{-8}$ M) induce preB cell apoptosis; (3) in long-term cultures, bone marrow stromal cells, but not preB cells, express AhR mRNA and protein as determined by *in situ* hybridization, RT-PCR, and immunoblotting; (4) freshly isolated unfractionated bone marrow cells, but not purified bone marrow B cells, express AhR protein as assessed by immunohistochemistry; (5) α -naphthoflavone, a competitive AhR inhibitor and cytochrome P450 antagonist, completely blocks DMBA-induced preB cell apoptosis in primary bone marrow cultures; and (6) DMBA or benzo[a]pyrene injection *in vivo* results in bone marrow cell apoptosis consistent with the death of hematopoietic cells clustered around stromal elements. The results implicate programmed cell death as a mechanism underlying DMBA-mediated immunosuppression and suggest that preB cell death is influenced by local interactions with AhR⁺ bone marrow stromal cells. © 1997 Academic Press

For many years research with polycyclic aromatic hydrocarbons (PAH)² has focused on the ability of these common environmental chemicals to induce cell transformation. More recently PAH have been investigated because of their more acute induction of a number of biologic responses including immunosuppression (Burchiel *et al.*, 1992, 1993; Davila *et al.*, 1995; Hardin *et al.*, 1992; Hinoshita *et al.*, 1992; Kawabata and White 1987; Thurmond *et al.*, 1988; White and Holsapple 1984; White *et al.*, 1985; Wojdani *et al.*, 1984; Yamaguchi *et al.*, 1996; Temple *et al.*, 1993). Several PAH, including 7,12-dimethylbenz[a]anthracene (DMBA), benzo[a]pyrene (B[a]P), and fluoranthene, as well as related halogenated hydrocarbons like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs), induce thymic atrophy, decrease resistance to infectious agents and transplantable tumors, reduce bone marrow cellularity, alter lymphocyte homing, impair B and T lymphocyte proliferative responses, inhibit B cell antibody responses, decrease cytotoxic T cell activity, induce cell death in myeloid, B, and T cells, inhibit natural killer activity, or decrease cytokine production in animal model systems (Davis and Safe, 1991; Dooley and Holsapple 1988; Fine *et al.*, 1990; Gasiewicz and Rucci 1991; Greenlee *et al.*, 1985; Harper *et al.*, 1995; Holladay and Smith 1995; Holsapple *et al.*, 1991; Kerkvliet *et al.*, 1990; Kremer *et al.*, 1994; Morris *et al.*, 1994; Wood *et al.*, 1993; Ackerman *et al.*, 1989; Fine *et al.*, 1990; Kerkvliet *et al.*, 1990; Silkworth *et al.*, 1984; Burchiel *et al.*, 1992; White and Holsapple, 1984; Karras and Holsapple, 1994). Epidemiological studies suggest that PAH are immunosuppressive in humans as well (Hoffman *et al.*, 1986; Kimbrough, 1987; Szczeklik *et al.*, 1994). Although the intracellular mechanisms by which PAH suppress immunity are ill-defined, some studies support a role for the aryl hydro-

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² Abbreviations used: AhR, aryl hydrocarbon receptor; B[a]P, benzo[a]pyrene; DMBA, 7,12-dimethylbenz[a]anthracene; PAH, polycyclic aromatic hydrocarbon(s); PI, propidium iodide; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

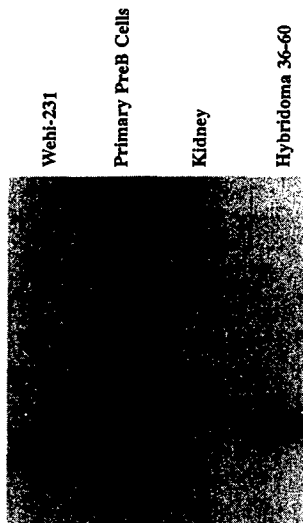


FIG. 1. Long-term bone marrow cultures contain preB cells. Lymphoid cells (>90% CD45/B220⁺) were harvested from adherent monolayers of primary bone marrow stromal cells. DNA was extracted from these cells, WEHI-231 (B cell lymphoma) cells, kidney cells, and 36-60 B cell hybridoma cells, electrophoresed, and blotted with a JH probe. Germ-line IgH genes are evident in kidney cells. Rearranged IgH genes are present in clonal WEHI-231 and 36-60 hybridoma cells. A smear of rearranged IgH genes in B cells from bone marrow cultures is characteristic of a population of preB cells.

carbon receptor (AhR) and/or PAH metabolism in immunosuppression (Hardin *et al.*, 1992; Holsapple *et al.*, 1991; Kerkvliet *et al.*, 1990; Ladics *et al.*, 1991; Morris *et al.*, 1994; Silkworth *et al.*, 1984; Thurmond *et al.*, 1988; White *et al.*, 1985; Harper *et al.*, 1994).

Relatively few studies have evaluated the effects of PAH or related halogenated hydrocarbons on developing immune systems (Morris *et al.*, 1994; Blaylock *et al.*, 1992; Luster *et al.*, 1988), an important consideration given the comparative sensitivity of developing biologic systems to environmental pollutants (Holladay and Smith, 1995). In the present study, the ability of a prototypic PAH, DMBA, to alter lymphocyte growth in an *in vitro* model of B lymphopoiesis was studied. Our investigations were motivated by studies demonstrating DMBA-induced suppression of mature lymphocyte activity *in vivo* and *in vitro* (Holladay and Smith, 1995; Burchiel *et al.*, 1992, 1993; Davila *et al.*, 1995; Ladics *et al.*, 1991).

As *in vivo*, growth and development of bone marrow-derived preB cells in long-term bone marrow cultures (Whitlock *et al.*, 1984) are dependent on contact with, and growth factor production by, bone marrow stromal cells (Pietrangeli *et al.*, 1988). Thus, adverse effects of DMBA on preB cell populations could reflect direct effects on the preB cells themselves and/or stromal cells on which preB cells depend for growth support. In the present studies, particular emphasis was placed on identifying the mechanism(s) of DMBA immunotoxicity, the possible role of the AhR or PAH metabolism in biologic responses to DMBA, and the potential for

DMBA immunotoxicity to be mediated by modification of bone marrow stromal cell function, rather than by direct effects on immature B lymphocytes.

MATERIALS AND METHODS

Bone marrow cultures. Murine bone marrow cultures were prepared from C57BL/6 (AhR^b) bone marrow as described (Whitlock *et al.*, 1984). Briefly, bone marrow was expunged from the femurs of 3- to 4-week-old mice, washed, counted, and cultured in RPMI medium containing 5% FCS (Gibco/BRL, Inc., Grand Island, NY), 2 mM L-glutamine (Gibco/BRL), 50 U/ml penicillin-streptomycin (Gibco/BRL), and 0.05 mM 2-mercaptoethanol (Mallinckrodt, Paris, KY) (3×10^6 cells/3 ml in 6-well plates). One-half of the medium was replaced every 3-4 days with fresh medium. Growth of stromal cells and stromal cell-adherent lymphocytes was routinely evident after 10 days. After 4 weeks of culture, >90% of stromal cell-adherent cells consisted of B220⁺, sIgM⁻ B lymphocytes as assessed by flow cytometry (see below) with rearranged immunoglobulin heavy chains (Fig. 1). To assess apoptosis, cultures were treated with vehicle (acetone) or various concentrations of DMBA dissolved in acetone (final vehicle concentration = 0.1%) in duplicate wells. At various points thereafter preB cells from duplicate wells were pooled and assayed for apoptosis. Vehicle had no effect on cultures.

Southern blot analysis of Ig heavy chain gene rearrangements. Procedures for Southern blotting and hybridization using the JH probe "P2" have been previously described in detail (Near and Haber, 1989) with the exceptions that charged nylon filters were used and that the DNA transfer

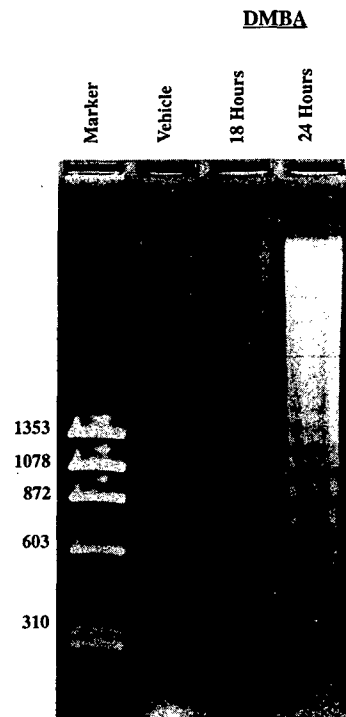


FIG. 2. DMBA induces DNA fragmentation in primary bone marrow cultures. Vehicle (0.1%) or DMBA (10^{-5} M) was added to 3- to 4-week-old bone marrow cultures in duplicate wells. Cells were harvested after 18 and 24 hr, DNA extracted, and electrophoresed in 3.5% agarose gels. Digestion of DNA into oligonucleosomal fragments (i.e., multiples of 200 base pairs) results in a ladder pattern characteristic of apoptosis.

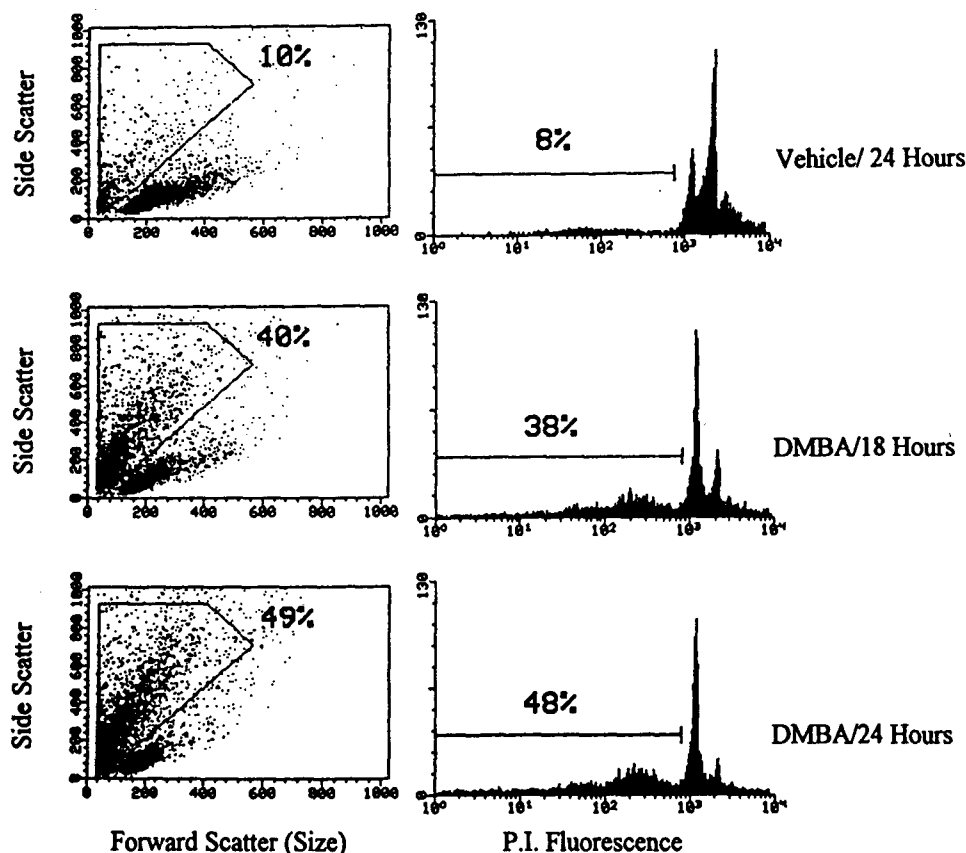


FIG. 3. DMBA induces morphologic and chromatin changes characteristic of apoptosis in primary preB cell populations. Vehicle (0.1%) or DMBA (10^{-4} M) was added to 3- to 4-week-old bone marrow cultures in duplicate wells. Cells were harvested after 18 and 24 hr. Cells were divided into two aliquots. Cells from one aliquot were treated with PI in hypotonic buffer for DNA analyses (histograms). Cells from the second aliquot were resuspended in PBS for forward (size) and side (granularity) light-scatter analyses (dot plots). Apoptotic cells stain poorly with PI (regions in histograms) and are relatively small and granular (enclosed region of each dot plot). A representative experiment is shown. Comparable results were obtained with lower doses of DMBA (see Fig. 4).

was done using alkaline transfer as described by the manufacturer (Gene Screen Plus, NEN Research Products, Boston, MA).

Fluorescence analyses and sorting. PreB cells were harvested by gently washing 3- to 4-week-old primary bone marrow cultures with media. To assess purity, preB cells were incubated for 40 min on ice with phycoerythrin-anti-CD45/B220 (clone RA3-6B2, rat IgG 2a, Pharmingen) or with a phycoerythrin-labeled isotype control. Cells were washed and analyzed in a Becton-Dickinson FACScan flow cytometer. To sort CD45/B220⁺ preB cells from bone marrow cultures or to sort bone marrow B cells from freshly isolated bone marrow, 10^6 cells were incubated as above with PE-anti-CD45/B220 or PE-conjugated isotype control antibody, washed in medium containing 20% FCS, and sorted with a Becton-Dickinson FACStar on the basis of lymphocyte morphology (forward and side light-scatter parameters) and expression of CD45/B220. Sorted cells were reanalyzed after sorting and consisted of >95% CD45/B220⁺ cells.

Quantitation of apoptotic cells was performed as previously described (Hardin *et al.*, 1992; Hinoshita *et al.*, 1992; Yamaguchi *et al.*, 1996). Cells were washed in cold PBS, pelleted, and resuspended in 0.5 ml of hypotonic fluorochrome solution containing 50 μ g/ml propidium iodide (PI; Sigma Chemical Co., St. Louis, MO), 1% sodium citrate, and 0.1% Triton X-100 (Sigma). Cells exhibiting DNA fragmentation and apoptosis were shown to be those in which PI fluorescence was weaker than the typical G₀/G₁ cell cycle peak. For analysis of cell morphology by flow cytometry, cells were resuspended in PBS containing 10% FCS.

DNA gels. Cells (10^6) were washed and resuspended in cold 10 mM Tris/1 mM EDTA (TE) buffer containing 0.2% Triton X-100. Debris was pelleted and supernatant transferred to a fresh tube. After addition of 35 μ l of 3 M sodium acetate, DNA was extracted with phenol-chloroform. Fragmented DNA in supernatants was precipitated with ethanol, pelleted, rinsed with cold ethanol, dried, and resuspended in Tris/EDTA buffer. For gel electrophoresis, samples were added to loading buffer consisting of 40% sucrose in Tris/EDTA buffer, 1% SDS (Sigma), bromophenol blue, and 2.5 μ g/ml RNase (Gibco/BRL) and loaded into dry wells of a 3.5% NuSieve agarose gel (FMC Bioproducts, Rockland, ME) in Tris-acetate buffer. Gels were run at 50 V for 2 hr and stained with ethidium bromide.

AhR immunoblotting. PreB cells were gently washed off bone marrow stromal cell monolayers and transferred to new culture wells for 3 hr before harvest to minimize contamination with plate-adherent stromal cells. Stromal cells were lifted from plates by a 3-min treatment with 0.25% trypsin containing 1 mM EDTA·4Na (Gibco-BRL). Cells were washed twice in cold PBS, resuspended in lysing buffer (1% Triton X-100, 150 mM NaCl, 25 mM Tris-HCl, 1 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM EDTA, 50 mM NaF, 1 mM orthovanadate, 1 mM PMSF), and centrifuged for 15 min at 15,000g. Protein concentrations in supernatants were measured with a bicinchoninic acid protein assay reagent kit (Pierce Chemical Co., Rockford, IL). Samples were diluted in Laemmli buffer and loaded into 7.5% SDS-polyacrylamide gels. Electrophoresis was carried out at 150 V for 1 hr. Proteins were transferred from gels to nitrocellulose filters (Bio-Rad, Her-

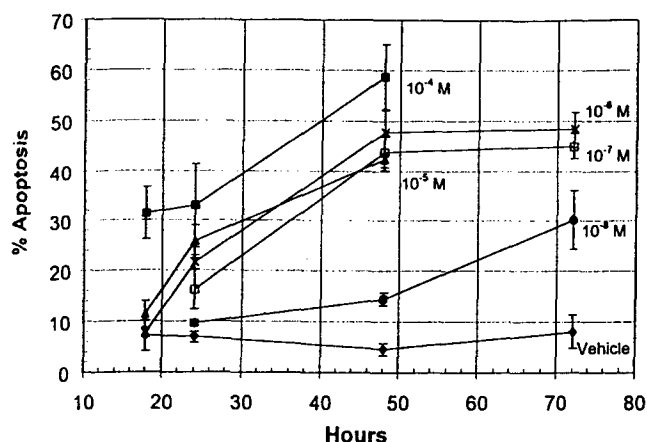


FIG. 4. Kinetics and dose response of primary preB cells to DMBA. Vehicle (0.1%) or DMBA (10^{-4} – 10^{-8} M) was added to 3- to 4-week-old bone marrow cultures. Eighteen to 72 hr later preB cells were harvested and stained with PI, and the percentage of cells undergoing apoptosis was quantitated by flow cytometry. Each data point represents data obtained from three to four experiments. Apoptosis following DMBA exposure reached statistical significance relative to vehicle controls ($p < 0.05$) at 18 hr with 10^{-4} M, at 24 hr with 10^{-6} M, and at 48 hr with 10^{-8} M DMBA.

cules, CA) at 150 V for 1 hr or at 30 V overnight. Efficiency of transfer was monitored by staining proteins with 0.1% Ponceau S (w/v) in 5% acetic acid (v/v) (Sigma). Ponceau S was washed out with double-distilled water followed by TBST buffer (20 mM Tris, 0.5 M NaCl, 0.03% Tween 20, pH 7.5). Filters were blocked with TBST buffer containing 5% dry milk, washed twice for 5 min in TBST, and incubated with monoclonal anti-AhR antibody Rpt1 (Singh and Perdew, 1993) at a 1:10,000 dilution for 1 hr at room temperature. Filters were washed three times with TBST and incubated for 1 hr at room temperature with a 1:6000 dilution of HRP-goat anti-mouse antibody (Sigma). Filters were washed twice and developed by chemiluminescence (Du Pont NEN Research Products Co., Boston, MA).

Immunohistochemistry. Freshly isolated bone marrow cells or CD45/B220⁺ cells were cytospun onto glass microscope slides, air dried, and fixed in 10% formalin. Slides were then incubated with 2 μ g/ml rabbit polyclonal anti-AhR antibodies or control rabbit immunoglobulin for 1 hr at 37°C and washed and incubated with a 1:3000 dilution of biotinylated swine anti-rabbit immunoglobulin antibody for 30 min at 37°C. Background peroxidase activity was quenched with 3% H_2O_2 for 10 min, cells were washed, and horseradish peroxidase-conjugated streptavidin was added for a 25-min incubation at 37°C. AhR-specific staining was visualized by incubating slides in 0.1% 3,3'-diaminobenzidine and 0.04% H_2O_2 (DAKO, Carpinteria, CA) in PBS solution for 10 min at room temperature. Slides were then washed in running tap water and counterstained with hematoxylin, coverslipped with Permount, and examined by light microscope. AhR staining under these conditions was completely inhibited by absorbing anti-AhR antibody with Sepharose beads conjugated with recombinant AhR protein. The stain was not affected by absorbing anti-AhR antibody with Sepharose beads conjugated with an irrelevant protein, lysozyme (data not shown).

RT-PCR for AhR mRNA. PreB cells were washed off stromal cell monolayers by vigorous pipetting and RNA prepared from 5×10^6 cells

as described (Leedo Medical Laboratories, Houston, TX). Adherent stromal cells ($3-5 \times 10^6$) were lysed directly in tissue culture wells. Integrity of RNA samples was assayed by electrophoresis in 1.5% agarose gels prior to RT-PCR to detect AhR mRNA in 5- μ g samples (total RNA) as described (SuperScript Preamplification System; Gibco/BRL). cDNA was amplified for 35 cycles with $MgCl_2$ concentration adjusted to 2.5 mM to maximize specific signal and using the following AhR primers: CTGGCAATGAAT-TTCCAAGGGAGG and CTTTCTCCAGTCTTAATCATGCG. Primers were chosen to enclose the sequence which contains the putative murine AhR ligand-binding domain (Dolwick *et al.*, 1993; Ema *et al.*, 1992). Amplified DNA was electrophoresed through 3% gels (3:1 NuSieve:LE agarose; FMC, Rockland, ME) and DNA visualized by ethidium bromide staining. All samples were normalized for equal loading of gels with the GADPH housekeeping gene. GADPH mRNA was reverse transcribed and cDNA amplified with the following primers: CCATCACCATCTTCCAGGAG and CCTGCTTCACCACCTTCTTG.

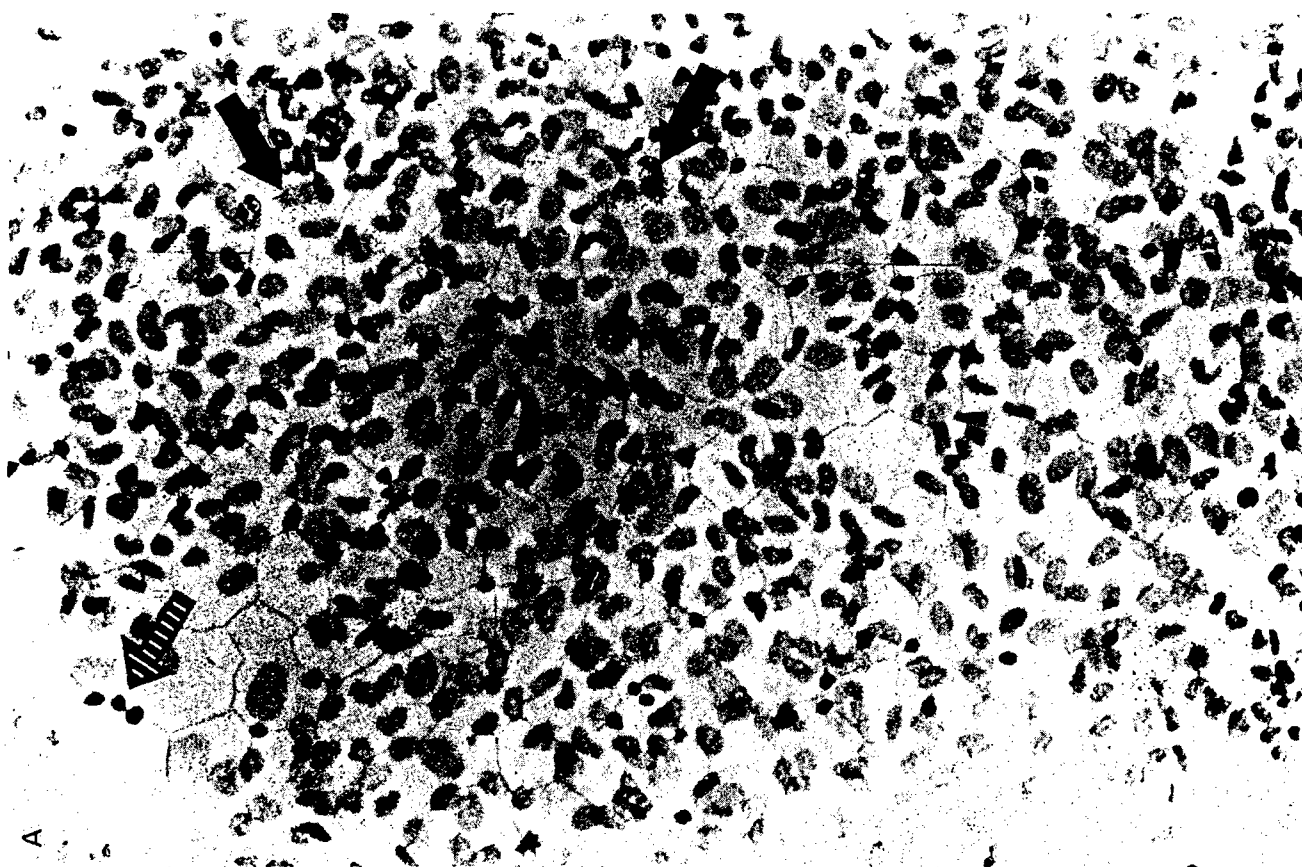
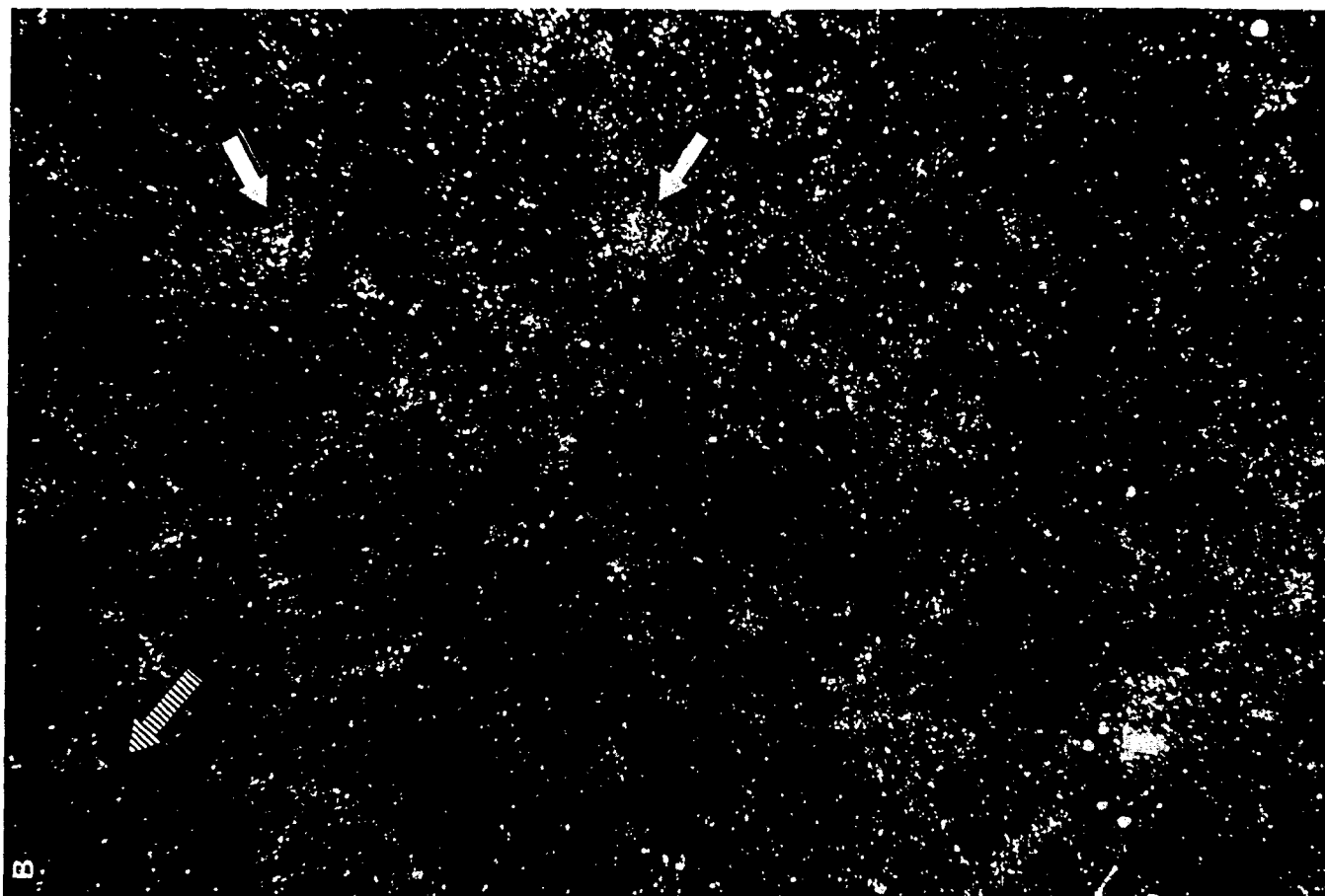
In situ hybridization. 35 S-radiolabeled AhR riboprobes were generated using T7 (sense) and SP6 (antisense) promoters with linearized *Xba*I and *Hind*III digests of pcDNA-AhR murine AhR cDNA as template. PreB cells harvested from 4-week-old bone marrow cultures were fixed with 4% paraformaldehyde, dehydrated, and hybridized for 18 hr at 52°C with either sense or antisense riboprobe. Slides were washed and autoradiographs exposed for 6 weeks. No signal was observed with the sense riboprobe.

Terminal deoxynucleotide transferase (TdT) labeling for in situ analysis of apoptosis. In situ analysis of DNA integrity was assessed using the protocol of Tilly *et al.* (1995). Bone was fixed and decalcified (Surgipath, Richmond, IL) for 2 days, changing the solution each day. Tissue was rinsed in water for 1 hr, embedded in paraffin, sectioned, and mounted unstained onto microscope slides. Paraffin was removed by incubating for 30 min in a 60°C oven followed by a 10-min wash in xylenes. Tissue was rehydrated with graded concentrations of ethanol as follows: 100% ethanol for 10 min, 90% ethanol for 3 min, 2% H_2O_2 in 90% methanol for 1 min, 80% ethanol for 3 min, 70% ethanol for 3 min, and sterile water for 3 min. Each section was treated with proteinase K (10 μ g/ml in 2 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4) and incubated for 30 min at 37°C. Slides were washed twice with sterile water. Tissue was preequilibrated with 5 \times TdT reaction buffer provided by the manufacturer and $CoCl_2$ (Boehringer-Mannheim, Indianapolis, IN) for 20 min. Slides were then incubated for 15 min at 37°C with the reaction mixture for 3'-end labeling. The reaction mixture consisted of TdT reaction buffer, 5 mM $CoCl_2$, 50 μ M biotin-16-dUTP, 500 μ M dUTP, and 25 units/0.1 ml TdT enzyme (Boehringer-Mannheim). Slides were washed three times for 10 min each in 150 mM NaCl, 100 mM Tris-HCl (pH 7.5) buffer and then blocked by incubation with 3% BSA (w/v) in buffer for 30 min at room temperature. ABC reagent (Vector Labs, Inc., Burlingame, CA) was added, and slides were incubated at room temperature for 15 min, dipped in buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, and 50 mM $MgCl_2$), and washed three times for 10 min each in buffer. DAB (Vector Labs) was used to detect localization of incorporated biotin-dUTP in apoptotic cells. Reactions were stopped with TE buffer and slides counterstained with hematoxylin, followed by a lithium carbonate wash. Sections were dehydrated with a graded ethanol series (70–100% ethanol; 1 min each) and allowed to air dry before coverslip mounting with Permount.

RESULTS

DMBA induces preB cell apoptosis. Culture of bone marrow cells under conditions described originally by Whit-

FIG. 5. In situ hybridization for AhR mRNA. Primary bone marrow cultures were fixed with 4% paraformaldehyde, dehydrated, and hybridized for 18 hr at 52°C with an AhR antisense 35 S-labeled riboprobe. Slides were washed and autoradiographs exposed for 6 weeks. Solid arrows indicate stromal cells expressing AhR mRNA signal. Striped arrows indicate preB cells. No signal was observed with AhR sense riboprobes (not shown). (A) Bright field. (B) Darkfield.



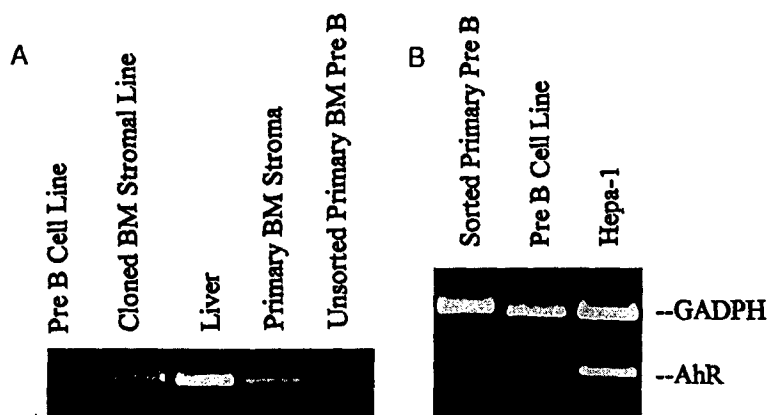


FIG. 6. RT-PCR for AhR mRNA in primary bone marrow stromal and preB cells. (A) RNA was extracted from a preB cell line (Yamaguchi *et al.*, 1996), a cloned bone marrow stromal cell line (Pietrangeli *et al.*, 1988), liver cells from C57BL/6 mice, bone marrow stromal cells from 4-week-old bone marrow cultures, or preB cells harvested from 4-week-old bone marrow cultures (90% CD45/B220⁺). RNA was reverse transcribed and cDNA amplified with AhR- and GADPH-specific primers. Samples were normalized according to the GADPH signal. The predominant AhR band is shown. (B) RNA was extracted from fluorescence-activated cell-sorted preB cells from 4-week-old bone marrow cultures (>95% CD45/B220⁺), a preB cell line (Yamaguchi *et al.*, 1996) and Hepa-1c17 hepatoma cells (Cuthill *et al.*, 1987). RNA was reverse transcribed and cDNA amplified simultaneously with AhR- and GADPH-specific primers.

lock *et al.* (1984) resulted in the outgrowth of CD45/B220⁺, slg⁻ B cell populations expressing rearranged immunoglobulin heavy chain genes, i.e., preB cell populations (Fig. 1). It was noted that addition of 10^{-5} M DMBA to these cultures resulted in the disappearance of preB cells within 7 days (data not shown). Examination of preB cells from DMBA-treated cultures by light microscopy revealed cellular and nuclear condensation characteristic of apoptosis. To confirm that DMBA induces preB cell apoptosis, primary bone marrow cultures were treated with vehicle or with 10^{-5} M DMBA. PreB cells were harvested 18 and 24 hr later, and DNA was extracted for analysis of fragmentation characteristic of apoptosis (Ju *et al.*, 1995). A low but discernible level of digestion of DNA into oligonucleosomal (i.e., multiples of 200 base pairs) fragments was detected in control cultures (Fig. 2). Exposure of cultures to 10^{-5} M DMBA for 24 hr resulted in a dramatic increase in DNA fragmentation. In other experiments not shown, increased DNA fragmentation was evident within 18 hr of DMBA exposure. These results are consistent with DMBA-mediated preB cell apoptosis.

To quantitate apoptosis, cultures were treated with vehicle or DMBA. PreB cells were harvested 18 to 24 hr later and the proportion undergoing apoptosis was quantitated by DNA staining with propidium iodide and by flow cytometric analyses of cell morphology (Hardin *et al.*, 1992). Data from one experiment (from over 20 total) are presented in Fig. 3. Relatively few (8%) vehicle-treated cells exhibited a dull PI staining pattern characteristic of cells undergoing apoptosis. Similarly, few vehicle-treated cells (10%) exhibited the classic morphologic features of apoptotic cells, i.e., smaller (lower forward scatter) and more granular (increased side scatter). The percentage of apoptotic cells, as defined by both morphologic and DNA staining parameters, increased

to approximately 40 and 50% following DMBA exposure for 18 and 24 hr, respectively. In contrast, bone marrow stromal cells were resistant to DMBA, demonstrating no change in PI staining 24 hr after DMBA exposure (data not shown). Collectively, the data confirm that DMBA induces preB cell apoptosis in primary bone marrow cultures.

Extensive kinetics and titration experiments indicated that significant levels of apoptosis, as assessed both by the PI staining pattern and by morphologic criteria (forward and side light-scatter parameters) were induced after 24 hr with as little as 10^{-6} M DMBA and after 48 hr with as little as 10^{-8} M DMBA (Fig. 4, $p < 0.05$). The percentage of cells undergoing apoptosis continued to rise 72 hr after exposure to 10^{-8} M DMBA.

Expression of AhR mRNA and protein in bone marrow cultures. Since the AhR has been implicated in PAH-in-

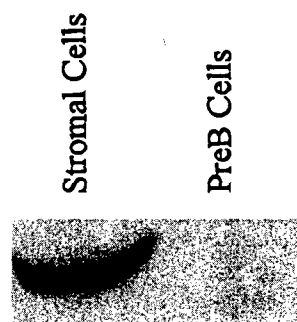
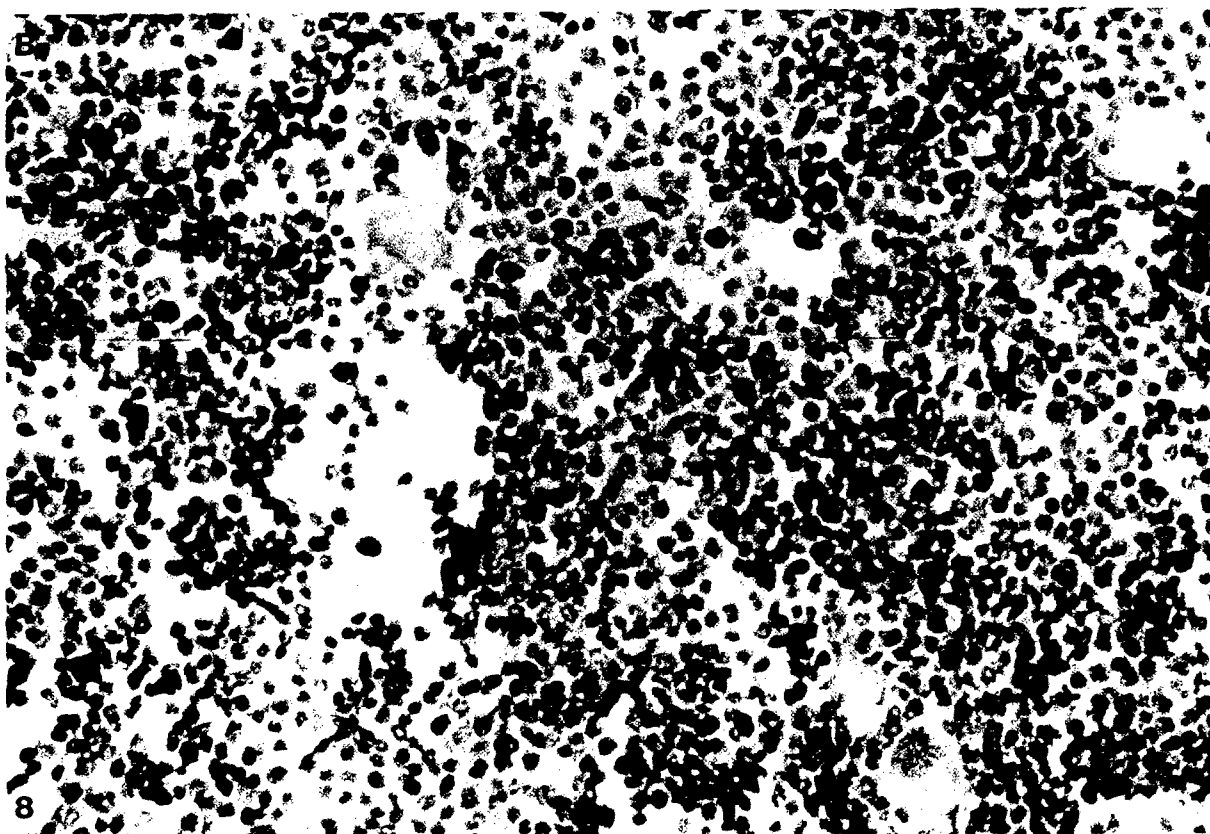
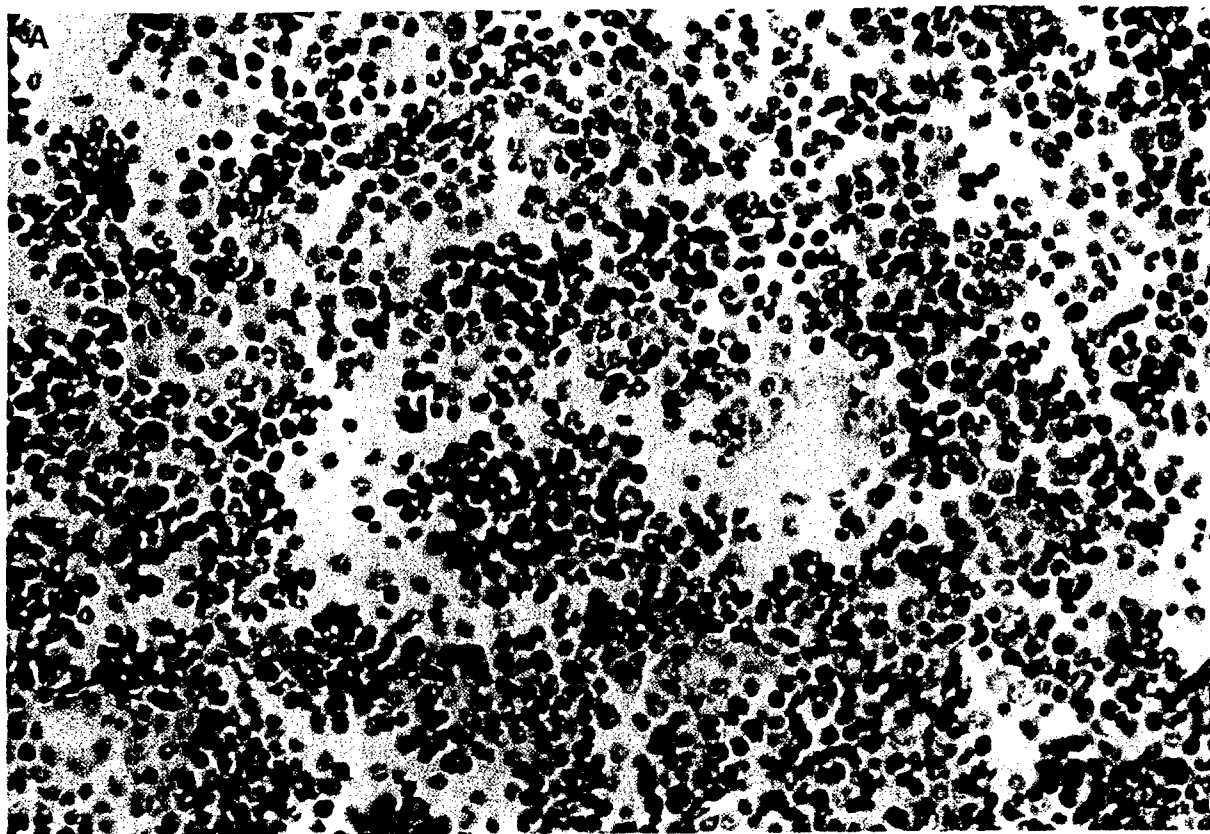
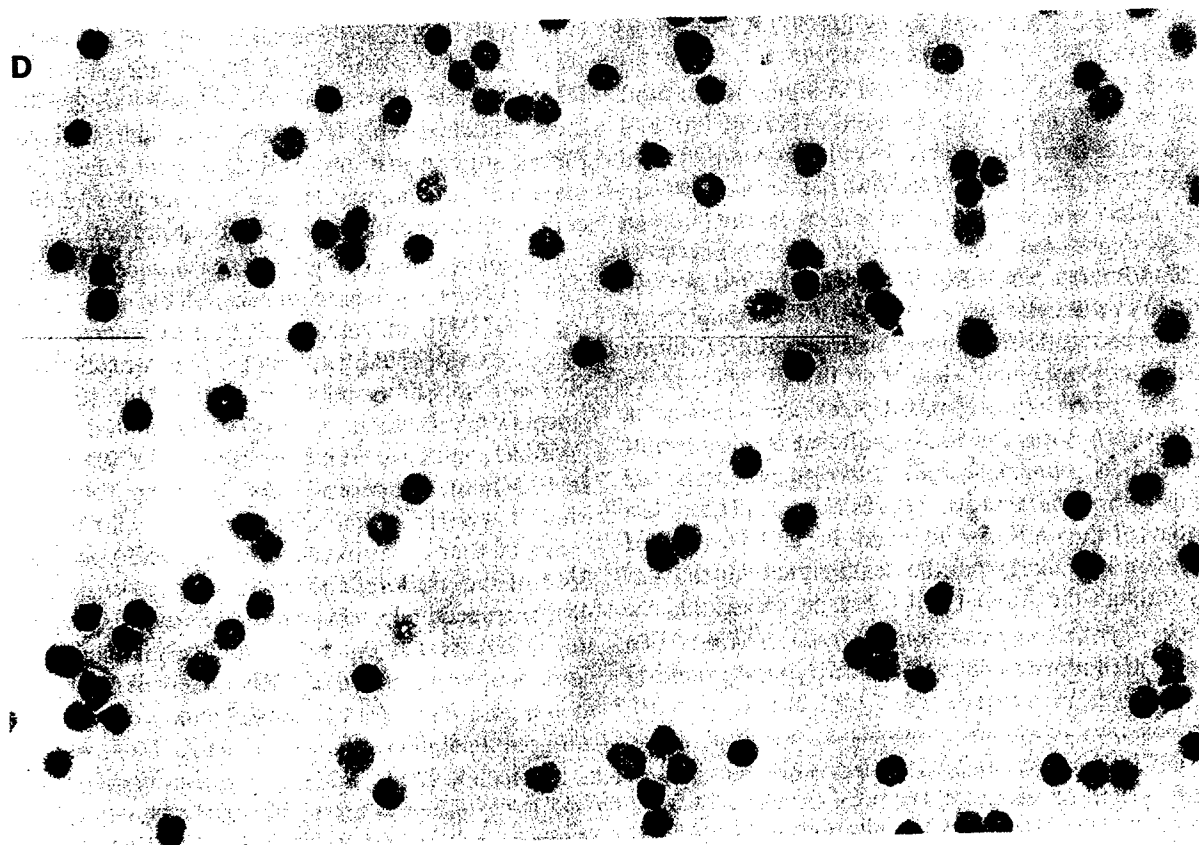
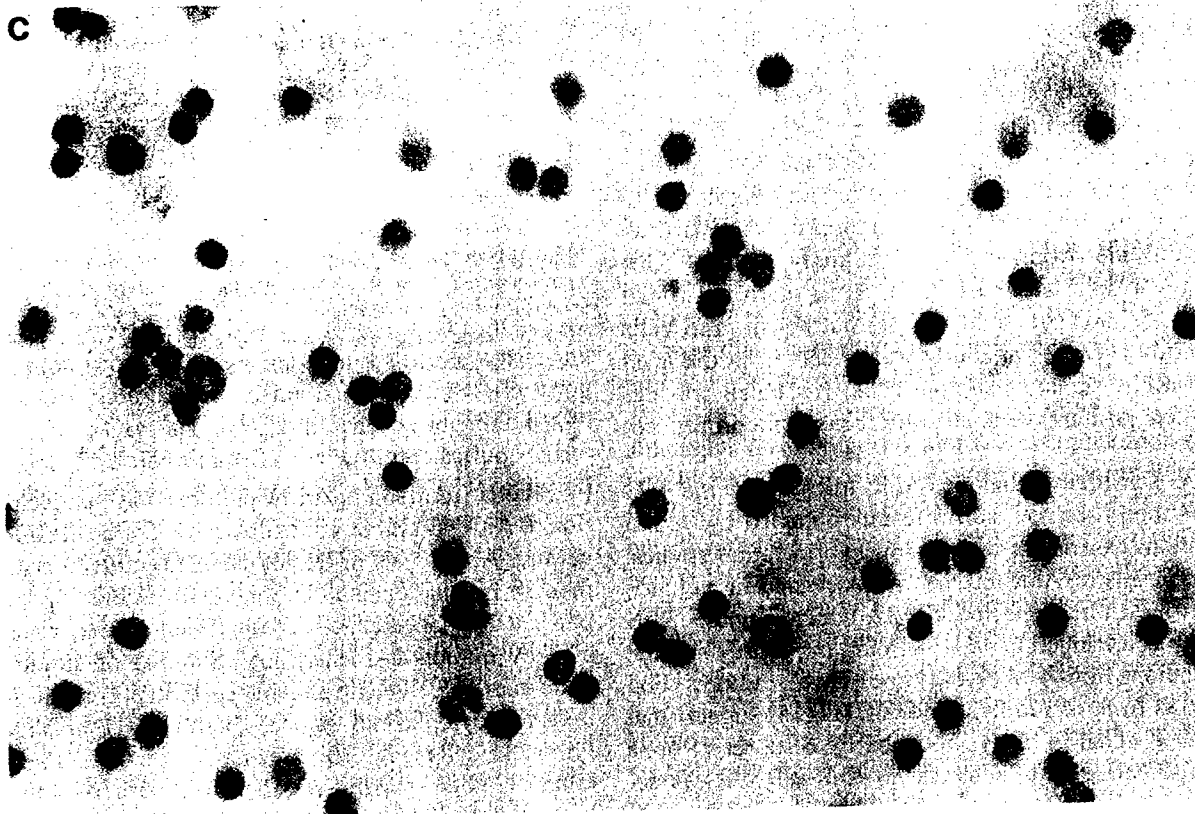


FIG. 7. Immunoblot for AhR in bone marrow stromal and preB cells. Total cellular protein from untreated stromal cells and preB cells from 4-week-old bone marrow cultures was extracted, electrophoresed, transferred to nitrocellulose filters, and immunoblotted with monoclonal (RPT-1) anti-AhR antibody. The predominant 95- to 100-kDa AhR band is shown. Data from a representative experiment are presented.





duced immunosuppression (Hardin *et al.*, 1992; Kerkvliet *et al.*, 1990; Morris *et al.*, 1994; Silkworth *et al.*, 1984; Thurmond *et al.*, 1988; White *et al.*, 1985; Wojdani *et al.*, 1984; Harper *et al.*, 1994), and since little is known about AhR expression either in immature lymphocyte populations or in the stromal cells which in part constitute the bone marrow microenvironment, *in situ* hybridization was employed to evaluate AhR mRNA expression in cultures of bone marrow-derived preB and stromal cells. Interestingly, a strong AhR mRNA signal was detected in some bone marrow stromal cells (Figs. 5A and 5B, solid arrows) but not in preB cells (striped arrow). Great variability was observed in the level of AhR mRNA signal in stromal cells. No signal was detected when probing with an AhR sense riboprobe (not shown).

A more sensitive technique, AhR mRNA-specific RT-PCR, was then used to confirm results obtained by *in situ* hybridization. Consistent with *in situ* hybridization studies, significant levels of AhR mRNA were detected in a cloned bone marrow stromal cell line, BMS2, which supports preB cell growth (Pietrangeli *et al.*, 1988) and in primary stromal cell populations from 4-week-old bone marrow cultures (Fig. 6A). Results from *in situ* hybridization (Fig. 5) suggest that most of that signal is likely produced by a subset of bone marrow stromal cells. In contrast, no AhR mRNA was detected in a stromal cell-dependent preB cell line (Yamaguchi *et al.*, 1996) and minimal AhR mRNA was detected in unsorted primary preB cell populations harvested from bone marrow cultures (Fig. 6A).

To determine if this weak AhR mRNA signal in the preB cell population was due to contamination with AhR⁺ stromal cells, preB cells from bone marrow cultures were stained for CD45/B220 expression and sorted on the basis of forward (size) and side (morphology) light-scatter parameters and CD45/B220 expression. Sorted B cell populations were >95% CD45/B220⁺. No AhR mRNA was detected in these sorted primary preB cell populations (Fig. 6B), strongly suggesting that preB cells from bone marrow cultures do not express AhR.

Western immunoblotting for AhR protein was consistent with AhR mRNA assays in that significant levels of AhR were detected in primary stromal cells but not preB cell populations from bone marrow cultures (Fig. 7). These data suggest that if the AhR is involved in DMBA-induced apoptosis, then it is likely that the death signal is delivered indirectly through the stromal cell feeder layer.

Expression of AhR in fresh bone marrow isolates. To confirm that the failure to detect AhR mRNA or protein in

TABLE 1
α-Naphthoflavone Blocks DMBA-Induced Apoptosis^a

Time	Vehicle	α-NF	DMBA	α-NF + DMBA
24 hr	6 ± 2	9 ± 1	23 ± 8	12 ± 3*
48 hr	8 ± 2	10 ± 1	35 ± 1	8 ± 2*

^a Vehicle, 10⁻⁵ M α-naphthoflavone (α-NF), and/or 10⁻⁴ M DMBA were added to bone marrow cultures 3 weeks after culture initiation. PreB cells were harvested 24 or 48 hr later and the percentage of cells undergoing apoptosis was quantitated by the P.I., flow cytometry method. Data pooled from four experiments are presented as percentages apoptosis ± standard error. An asterisk indicates a significant decrease in percentage apoptosis *p* < 0.05 (*t* test).

preB cells from bone marrow cultures was not due to selection of AhR⁻ preB cells or to down-regulation of AhR during culture, AhR levels in freshly isolated, unfractionated bone marrow populations and in bone marrow B cell populations purified by fluorescence-activated cell sorting were evaluated by immunohistochemistry. Staining of unfractionated bone marrow cells with an AhR-specific polyclonal antibody revealed cytoplasmic staining, particularly in large, non-lymphoid cells (Fig. 8B vs negative control in Fig. 8A). In contrast, bone marrow B cells, purified by sorting on the basis of forward and 90° light-scatter parameters and CD45/B220 expression, were not stained with anti-AhR antibody (Fig. 8D vs negative control in Fig. 8C). In addition, no AhR mRNA was detected in sorted bone marrow B cells by RT-PCR (data not shown). These results are consistent with those obtained with long-term bone marrow cultures and further suggest that any AhR-dependent effects on this immature B cell population are dependent on AhR⁺, non-B cells in the bone marrow microenvironment.

α-Naphthoflavone blocks DMBA-induced preB cell apoptosis in bone marrow cultures. If the AhR and/or AhR-regulated P450 enzymes are critical to generation of a death signal, then it would be predicted that α-naphthoflavone (α-NF), a competitive inhibitor of the AhR (Gasiewicz and Rucci, 1991; Blank *et al.*, 1987; Merchant *et al.*, 1993) and P450IA1 inhibitor (Gurtoo *et al.*, 1979), would block DMBA-induced apoptosis. As shown in Table 1, addition of 10⁻⁵ M α-NF had no effect on preB cell viability. In this series of four experiments, 10⁻⁴ M DMBA induced 23 and 35% of the preB cells to undergo apoptosis within 24 and 48 hr, respectively. However, addition of a log less α-NF to DMBA-treated cultures completely rescued preB cells from apoptosis (*p* < 0.05). This result is consistent with

FIG. 8. Immunohistochemical analysis of AhR in fresh bone marrow cells. Bone marrow cells were expunged from the femurs of C57BL/6 mice and stained for CD45/B220 expression. Cells were sorted on the basis of forward and 90° light-scatter parameters (i.e., lymphocyte gated) and CD45/B220 fluorescence. Unsorted and sorted populations were cytopspun onto glass slides, fixed, and stained for AhR protein expression. (A) Unsorted cells, immunoglobulin control; (B) unsorted cells, anti-AhR antibody; (C) CD45/B220 sorted cells, immunoglobulin control; (D) CD45/B220 sorted cells, anti-AhR antibody.

role for the AhR in preB cell death. It is also possible that the inhibitory effect of α -NF reflects a contribution of P450 enzyme activity to apoptosis. Since the AhR influences P450 activity (Cuthill *et al.*, 1987; Dolwick *et al.*, 1993; Ema *et al.*, 1992), a role for P450 would still be consistent with the ability of the AhR to influence DMBA-induced apoptosis in immature B lymphocytes.

PAH induce bone marrow cell apoptosis in vivo. To begin to extend studies from this model system of B lymphopoiesis to the bone marrow environment *in vivo*, mice were injected ip with vehicle, B[a]P, or DMBA. Eighteen and 48 hr later, mice were sacrificed, and femurs were removed and analyzed for apoptotic cells using an *in situ* DNA labeling assay. In this assay, apoptotic cells can be identified both by morphologic criteria (small cells with nuclear condensation and/or nuclear apoptotic bodies) and by TdT-catalyzed biotinylated nucleotide incorporation (visualized as a dark blue-brown stain). Bone marrow from vehicle-treated mice contained few apoptotic cells 18 hr (Fig. 9A) or 48 hr (Fig. 9D) after vehicle injection. In contrast, bone marrow from B[a]P- or DMBA-treated mice exhibited significant numbers of apoptotic cells as early as 18 hr after treatment (Figs. 9B and 9C, arrowheads) and persisting at least until 48 hr (Figs. 9E and 9F). In most sections, apoptotic cells tended to occur in clusters (Figs. 9B and 9C). Occasionally, apoptotic cells could be visualized in apparent association with stromal cells (Fig. 9C, large arrowhead). In agreement with previous reports (Nebert *et al.*, 1980), significant hypocellularity was noted 48 hr after injection with B[a]P or DMBA (Figs. 9E and 9F vs control in Fig. 9D). These data are consistent with PAH-mediated induction of hematopoietic cell apoptosis *in vivo*.

DISCUSSION

DMBA acts as an immunosuppressant *in vivo* and alters lymphocyte function *in vitro* (Burchiel *et al.*, 1993; Burchiel *et al.*, 1992; Ladics *et al.*, 1991). In the present work we extend these studies with an investigation into the effects of DMBA on developing bone marrow lymphocytes. Having observed that DMBA inhibits growth of preB cells in long-term bone marrow cultures, we demonstrate herein that the mechanism of this immunosuppression is, at least in part, the induction of preB cell apoptosis.

Apoptosis is a manifestation of programmed cell death and is a critical process in development of the immune system. That is, upon ligation of antigen-specific receptors, immature autoreactive lymphocytes are deleted by activation of the cell death program (Ju *et al.*, 1995). Therefore, it is of significance that DMBA can activate the cell death program regardless of lymphocyte receptor specificity. Implications of this finding include a skewing of the developing lymphocyte repertoire and/or diminution of emerging lymphocyte populations. Suppression of lymphopoiesis could result in

enhanced susceptibility to infectious disease, particularly if generalizable to the T cell compartment. The latter possibility is currently under investigation.

Titration experiments demonstrate that significant levels of apoptosis are induced with as little as 10^{-8} M DMBA. DMBA-induced apoptosis is presented herein primarily as increasing percentages of cells exhibiting a sub G₀/G₁ peak, although it is also visualized by DNA fragmentation at doses as low as 10^{-8} M (Mann *et al.*, 1997), a corresponding decrease in cell size as assessed by flow cytometry, and nuclear condensation as seen by light microscopy. This PAH dose is lower than those generally reported to effect immunosuppression of mature lymphocyte populations *in vitro* (Burchiel *et al.*, 1993; Thurmond *et al.*, 1988; Ladics *et al.*, 1991), although a recent report demonstrates suppression of peripheral human lymphocyte mitogenic responses with 10^{-8} M DMBA (Davila *et al.*, 1996). Indeed, doses of DMBA capable of inducing apoptosis in the present system approach doses at which 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), generally considered to be the more potent xenobiotic, affects immune cell function (Harper *et al.*, 1995; Morris *et al.*, 1994; Luster *et al.*, 1988). Similarly, doses of B[a]P as low as 10^{-8} M suppressed B lymphopoiesis in bone marrow cultures (Hardin *et al.*, 1992). These results may be contrasted with those obtained with a noncarcinogenic PAH, benzo[*e*]pyrene, which binds the AhR poorly and which had no effect on B cells in bone marrow cultures at doses as high as 10^{-5} M (Hardin *et al.*, 1992). Finally, since techniques for measuring apoptosis assess cell death at a single time point, our studies may overestimate the doses of DMBA required to adversely affect immature lymphocytes in long-term cultures.

The ability of relatively low DMBA doses to effect B cell apoptosis may reflect the contribution of the stromal cell microenvironment to activation of the cell death program. That is, while DMBA doses on the order of 10^{-5} M are required to induce clonal B lymphoma cells to die (Burchiel *et al.*, 1993), concentrations of DMBA three log-orders less are required to kill preB cells maintained on bone marrow stromal cells. An important role for the lymphoid microenvironment in xenobiotic-induced immunosuppression has previously been suggested (Greenlee *et al.*, 1985; King *et al.*, 1989; Kremer *et al.*, 1994). Alternatively or in addition, the low doses required for DMBA-induced preB cell apoptosis may be due to heightened sensitivity of immature cells to xenobiotics in general.

The role of the AhR in immunosuppression of mature lymphocyte responses has been controversial (Morris *et al.*, 1994; Davis and Safe, 1991; Harper *et al.*, 1994, 1995; Thurmond *et al.*, 1988). Given the weight of evidence from both *in vivo* and *in vitro* studies (Kerkvliet *et al.*, 1990; Blank *et al.*, 1987; Morris *et al.*, 1994), it seems likely that at least part of the immunosuppression induced with AhR ligands is influenced by the AhR. Previous work from our laboratory

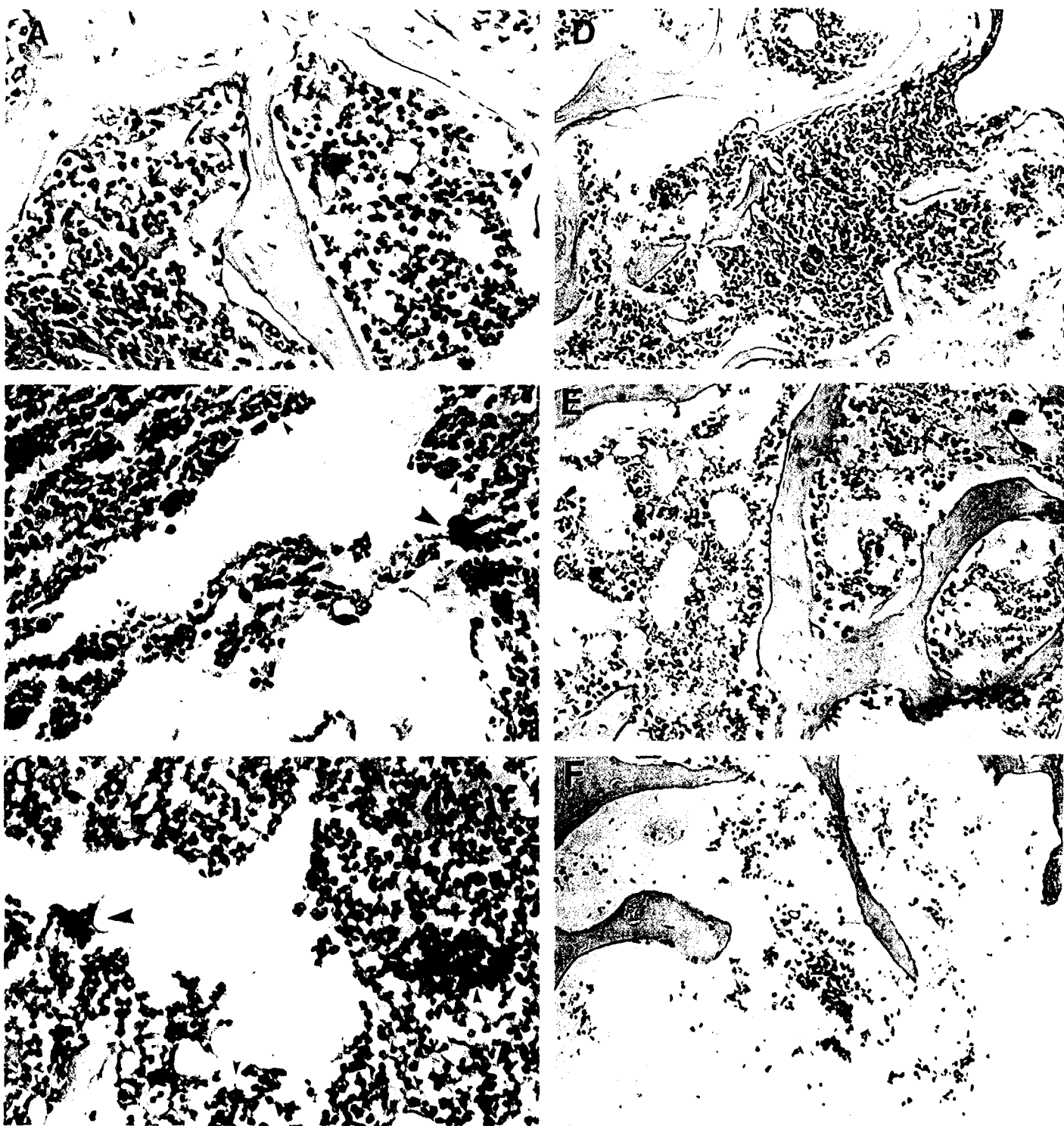


FIG. 9. *In situ* analysis of PAH-induced bone marrow cell apoptosis. C57BL/6 mice were injected ip with vehicle (0.2 ml) (A and D), 2 mg B[a]P (B and E), or 2 mg DMBA (C and F). Mice were sacrificed 18 (A-C; 200 \times) or 48 (D-F; 100 \times) hr later, and femurs were removed, fixed, decalcified, embedded, and analyzed for apoptotic cells *in situ* with the TdT assay. Apoptotic cells with condensed nuclei and/or DNA fragmentation (dark blue-brown TdT stain) are indicated with small arrowheads. Apoptotic cells frequently appear in clusters and sometimes in apparent association with stromal cells (large arrowheads).

has suggested a role for the AhR and/or AhR-regulated genes in B[a]P-mediated suppression of lymphopoiesis (Hardin *et al.*, 1992). Both B[a]P and DMBA induce α -NF-inhibitable AhR nuclear translocation and EROD activity (Yamaguchi *et al.*, 1997; Mann *et al.*, 1997). However, it remains to be determined in either the DMBA or B[a]P system if the AhR induces a death signal through second-messenger signaling (Gradin *et al.*, 1994; Enan and Matsumura, 1995), by inducing transcription of "death genes" or by indirectly influencing cell function through cytochrome P450 induction and the subsequent production of DMBA metabolites. The ability of nonlymphoid cells in the lymphoid microenvironment to metabolize PAH into compounds capable of suppressing mature lymphocyte responses is consistent with the latter possibility (Davila *et al.*, 1996; Ladics *et al.*, 1991). In any case, if the AhR and/or AhR-regulated P450 enzymes are important in PAH-induced apoptosis, then it is of particular significance that primary bone marrow stromal cells, but not preB cells from bone marrow cultures or freshly isolated bone marrow B cells, express AhR (Figs. 5–8). These results suggest a model in which low doses of AhR ligands, such as DMBA, activate AhR⁺ cells in the bone marrow microenvironment which in turn deliver signals to adjacent, immature B cells to activate their cell death program. Consistent with this hypothesis are the observations that a preB cell line maintained in stromal cell supernatant but in the absence of stromal cells or preB cells maintained on AhR⁻ feeder cells do not die in response to either DMBA or B[a]P (Near *et al.*, 1997). The role of the stromal cells is further supported by preliminary data demonstrating that a >10-kDa factor capable of inducing apoptosis in preB cells is elicited from the stromal cells after a 24-hr treatment with DMBA (data not shown).

It is not yet clear if the subset of stromal cells expressing AhR, and presumably responsible for preB cell apoptosis, represents a distinct cell type or a discrete stage in stromal cell development. Consistent with the latter possibility, our laboratory has recently demonstrated that mitogenic signals increase AhR expression in fibroblasts (Vaziri *et al.*, 1997). In either case, these results suggest a scenario in which lymphocytes contacting AhR⁺ stromal cell subsets undergo apoptosis following PAH exposure *in vitro*.

Since it is critical to eventually extend this model to whole animal studies, an *in situ* assay was employed to begin analysis of bone marrow cell apoptosis following *in vivo* administration of PAH. The advantage of such an assay is that it can localize individual apoptotic cells within the preserved architecture of the bone marrow environment, even when apoptotic cells are engulfed by phagocytic cells (Nakamura *et al.*, 1996). Confirmation of apoptosis using unfractionated bone marrow cell populations through gel electrophoresis or PI/FACS analyses would be problematic given the very rapid clearance of apoptotic cells *in vivo* and the prediction that apoptosis occurs primarily in a subset of cells associated

with stromal cells. Nevertheless, our results clearly demonstrate that bone marrow cells, many exhibiting lymphoid morphology, undergo apoptosis shortly after injection with either B[a]P or DMBA. The frequent clustering of apoptotic cells and the visualization of apoptotic cells in close proximity with epithelial-like stromal cells are consistent with, but do not prove, the hypothesis that lymphocyte apoptosis in response to PAH exposure *in vivo* is a localized phenomenon dependent on contact with AhR⁺ stromal cells. The challenge of experiments in progress is to perform detailed dose-response and kinetics studies *in vivo*, to definitively identify bone marrow cell subsets which undergo PAH-induced apoptosis *in vivo*, and to determine if AhR⁺ stromal cells are required for apoptosis *in vivo*, as appears to be the case *in vitro*.

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749

THE HUMAN 1,25-DIHYDROXYVITAMIN D₃ RECEPTOR AND PROKARYOTIC CHAPERONE PROTEINS FORM PROTEIN COMPLEXES. T.A. Craig and R. Kumar, Mayo Clinic/Foundation, Rochester, MN 55905.

Heat shock, or chaperone proteins, are involved in the function of some steroid/thyroid receptor family members. To investigate the role of chaperone proteins in human vitamin D receptor (VDR) function, we expressed full-length (FL; aa 1-427), DNA binding (DBD; aa 1-110) and ligand binding (LBD; aa 105-427) domains of hVDR in *E. coli BL21* cells as glutathione-S-transferase (GST) fusion proteins. Fusion constructs adsorbed to glutathione sepharose were washed extensively and proteins eluting with MgATP or glutathione were analyzed by SDS-PAGE, protein sequencing following transfer to PVDF and SDS-PAGE/immunoblotting (Western analysis) to detect proteins bound to the hVDR or glutathione S-transferase. The results are shown in the table below.

Proteins Associating with VDR Constructs and Eluting by MgATP Wash					
	GroEL	GroES	DnaK	*DnaJ	GrpE
FL VDR	++	-	++	+	+/-
DBD VDR	-	-	+	-	+/-
LBD VDR	-	-	++	+	+/-
GST	-	-	+	-	+/-

(-) none, (+) moderate, (++) large. *Not eluted by MgATP; remained bound to protein when assessed by SDS-PAGE following elution with glutathione.

All VDR constructs bound DnaK in amounts greater than or equal to GST. The FL hVDR bound specifically to GroEL and DnaJ whereas the LBD bound specifically to DnaJ. Our results show that the hVDR and domains thereof bind to bacterial chaperone proteins. Supported by NIH DK25409.

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20-EPI ANALOGS OF 1 α ,25(OH)₂D₃ AND CELL DIFFERENTIATION: MAP KINASE ACTIVATION & CONFORMATIONAL CHANGE OF 1 α ,25(OH)₂D₃ RECEPTOR. X. Song, E.D. Collins & A.W. Norman, Dept. Biochem., Univ. California, Riverside, CA 92521.

In the human acute promyelocytic leukemia cell line (NB4), 1 α ,25(OH)₂D₃ (with phorbol ester) stimulates alkaline phosphatase (ALP) expression (a marker for NB4 cell differentiation, Song & Norman, 1997). Rapid activation of mitogen-activated protein (MAP) kinase is involved in the action of 1 α ,25(OH)₂D₃ (Song, Bishop & Norman, 1997). We compared three 20-epi analogs with 1 α ,25(OH)₂D₃ on NB4 cell differentiation, MAP kinase phosphorylation and sensitivity of 1 α ,25(OH)₂D₃ receptor (VDR) to trypsin. All 20-epi analogs are 10- to 100-fold more potent than 1 α ,25(OH)₂D₃ on specific ALP induction, and 3- to 30-fold more potent on stimulation of MAP kinase phosphorylation. The *in vitro* sensitivity of VDR bound to 1 α ,25(OH)₂D₃ or 20-epi analogs to trypsin digestion were 10- to 100-fold different. We conclude: (1) the membrane-mediated MAP kinase activation is involved in 20-epi analog potentiation for NB4 cell differentiation; (2) 20-epi analogs can initiate different responses of MAP kinase phosphorylation; and (3) 20-epi analog-induced VDR conformation is different from that of 1 α ,25(OH)₂D₃, which may contribute to 20-epi potentiation of NB4 cell differentiation.

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ACTIVATION OF HUMAN ESTROGEN RECEPTOR BY TEA EXTRACTS

A. Traish, K. Murphy, L. Hafer, N. Savelyeva and A. Rogers.

Boston University School of Medicine Boston MA.

Epidemiological and experimental studies have linked intake of tea to reduced cancer risk at several sites. It has been suggested that growth of breast cancer cell lines is reduced by tea extracts. In this study, we investigated the effects of tea extracts on estrogen receptor (ER) binding and activation, as assessed by phosphorylation of ER on serine 118 in MCF-7 cells. (-)-Epigallocatechin (EGC), (-)-epigallocatechin-3-gallate (EGCG), theoflavin and green & black tea extracts displaced [³H] estradiol binding from ER. The order of effectiveness was EGCG > theaflavins = green tea polyphenols = black tea polyphenols > green tea powder > black tea powder. Dixon plot analyses of the data suggested that these compounds interact with ER binding site. Treatment of MCF-7 cells for 30 min at 37°C in culture with estradiol or diethylstilbestrol, but not progesterone or tamoxifen, resulted in ER phosphorylation, as did treatment with EGCG, theoflavin, green tea and black tea polyphenol extracts. These studies suggest that tea constituents possess moderate ER binding and estrogenic activity and may have physiological activity. The potential anti-carcinogenic effects of tea may be, in part, related to binding of tea polyphenols to ER and its phosphorylation. This work was supported by grants from DOD DAMD 17-94-J-4468 and DAMD 17-94-J-4421. Drs. D. Ballentine and S. Wheeler, Lipton, Inc., kindly provided the tea extracts.

750

EFFECTS OF CHRONIC ETHANOL ADMINISTRATION ON ESTROGEN RECEPTOR GENE EXPRESSION IN FEMALE RAT UTERUS. M. K. Mohamed and A. A. Abdel-Rahman, Department of Pharmacology, School of Medicine, East Carolina University, Greenville, NC 27858, USA.

In our previous studies, ethanol was found to attenuate the cardiovascular protective effects of estrogen. In this study, we investigated the effect of chronic ethanol administration on the gene expression level of estrogen receptor (ER- α) in an ER rich organ (rat uterus). Six groups of female Sprague-Dawley rats were used in this study. Two weeks before the ethanol administration, four groups were ovariectomized (OVX) and the remaining two groups were sham operated (SO). Estrogen (E2) timed release pellets (1.7 mg) was implanted subcutaneously in 2 groups of OVX rats (OVXE2). The other 4 groups (2 SO and 2 OVX) received placebo pellets. One group of each SO, OVX and OVXE2 received liquid diet containing 5% ethanol (daily intake approx. 10 g/kg), and respective control groups received isocaloric liquid diet. After 12 weeks, the rats were sacrificed, total RNA was prepared from the uterus, and quantitative RT-PCR was done using 18S ribosomal RNA as an internal standard (Quantum RNA™, Ambion, TX). Ethanol significantly increased the ER expression in SO rats. ER- α expression was significantly higher in OVX than in SO groups and was not affected further by the ethanol. On the other hand, E2 produced a significant decrease in ER- α in OVX rats compared with SO; an effect that was augmented in ethanol-fed OVX rats. These results suggest that the hormonal state influences the effect of chronically administered alcohol on ER- α expression in female rats.

Supported by Grant AA10257 from the NIAAA.

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THE USE OF UNDIFFERENTIATED PC12 CELLS AS A MODEL SYSTEM TO STUDY PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR) SIGNALING. J.W. Davis II, E.M. Mills, M.D. Kane and J.P. Vanden Heuvel (SPON: M.A. Belury), Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, National Institutes of Health, Bethesda, MD 20892, Parke-Davis, Ann Arbor, MI 48105 and Department of Veterinary Sciences, Penn State University, University Park, PA 16802.

The peroxisome proliferator-activated receptor (PPAR) is a steroid hormone receptor (SHR) that has been implicated in mediating the biological effects of peroxisome proliferators (PPs) such as fatty acids and leukotrienes. There has recently been a renewed interest in understanding upstream signaling events that modulate PPAR activity. Transient transfection experiments in COS-1 cells have demonstrated that signaling through the ras-raf signal transduction pathway results in increased ligand-mediated activation of the receptor as determined by the expression of a reporter molecule that contains the upstream promoter region of acyl-CoA oxidase (ACO), the prototypical target gene of PPAR. However, transient transfection assays are artificial systems that are often difficult to interpret. With this in mind we characterized the response of PC12 cells to Wy-14,643, a potent PP. PC12 cells were chosen because their signaling pathways are well characterized and because of the availability of numerous variants that express mutant signaling genes. This cell line expresses PPAR β predominantly and responds to PPs similarly to other model systems. Treatment with Wy-14,643 resulted in an increase in ACO message as determined by quantitative reverse transcriptase-PCR (RT-PCR). Furthermore, variants that overexpress dominant negative src or ras fail to respond to Wy-14,643. This data demonstrates the usefulness of these cells as a system to study PPAR function and underscores the connection between growth factor and SHR signal transduction pathways. (Supported by NIH DK49009 and NIH ES 07799)

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CLONING AND CHARACTERIZATION OF A MEMBER OF THE HUMAN RETINOL DEHYDROGENASE (RoDH) GENE FAMILY EXPRESSED IN SKIN. N. Markova¹, V. Jurukovski¹, K. Randolph^{1,2}, J. Napoli¹ and M. Simon^{1,2,3}. Department of Oral Biology & Pathology¹, Department of Dermatology², Living Skin Bank³, SUNY at Stony Brook, Stony Brook, NY 11794-8702 and Department of Biochemistry⁴, SUNY at Buffalo, Buffalo, NY 14214-3000.

Retinoic acid (RA) is an active metabolite that modulates gene expression through interactions with its nuclear receptors. In the target tissues RA alters cell proliferation and/or differentiation. The production of RA is tightly controlled, and in skin is a net product of retinol esterification/hydrolysis and oxido-reduction pathways. Deficient RA concentrations in the epidermis result in hyperkeratosis, while concentrations above 10⁻⁷M RA lead to reduced epidermal maturation and produce parakeratosis. The members of the retinol dehydrogenase gene family play a crucial role in the synthesis of the RA. By using primers derived from a conserved region of the known rat Retinol dehydrogenase (RoDH) genes and a PCR approach, we have cloned a human cDNA that belongs to the same family of transcripts. The deduced amino acid sequence shares 73% identity and 84% homology to the rat genes. Northern blot analysis showed that the gene is abundantly expressed only in the liver and the skin. In cultured epidermal keratinocytes the level of expression increases with the level of differentiation of the cultures. This finding correlates with the detection of mRNA by *in situ* hybridization predominantly in the spinous layers of the epidermis. Treatment of the cultures with RA results in a marked but transient induction of the mRNA levels, suggesting that contrary to the rat RoDH genes, the expression of this gene is under a complex regulation by retinoids.

2741

NUCLEAR FACTOR KAPPA B (NF- κ B) ACTIVATION IS INHIBITED AFTER ENDOTOXIN STIMULATION BY RESVERATROL, A NEW PHYTOLEXIN.

Minnie Q. Holmes-McNary, and Albert S. Baldwin, Jr. Lineberger Comprehensive Cancer Center, University of North Carolina-Chapel Hill, Chapel Hill, NC 27599-7295.

The cellular transcription factor, NF- κ B, mediates immune and inflammatory responses as well as regulate a diverse group of genes involved in cell proliferation, oncogenesis and apoptosis. The activation of these signal transduction pathways are regulated by protein tyrosine kinases (PTKs) and they have an important role in the regulation of transcription factors. More importantly, NF- κ B is under the control of PTKs. Considerable evidence indicate that after cellular stimulation NF- κ B translocation can be suppressed as well as NF- κ B dependent gene expression by PTK inhibitors. Resveratrol is a new phytoalexin found in grape skins and wines and is an inhibitor of PTKs. Recently, resveratrol was shown to inhibit cellular events associated with tumorigenesis and the inflammatory process. However, the mechanism of action for resveratrol has not been elucidated. Since NF- κ B mediates these responses, our studies focused on the ability of resveratrol to inhibit NF- κ B activity. We demonstrate that resveratrol inhibited LPS-induced NF- κ B DNA binding in THP-1 cells by EMSA. In addition, resveratrol treatment blocked LPS-induced activation of NF- κ B in a dose dependent manner. The inducible decay of I κ B was also prevented by resveratrol by Western blot. These studies indicate that resveratrol can regulate the nuclear transcription factor, NF- κ B, at the level of nuclear translocation. Further studies will determine whether resveratrol can inhibit NF- κ B mediated transactivation and transcription. Supported by the NIH-NRSA post-doctoral training grant.

2743

COMPARISON OF GENES EXPRESSED IN CHONDROCYTES GROWN IN MONOLAYER VS. THOSE GROWN IN SPINNER CULTURE USING SUBTRACTIVE HYBRIDIZATION R. Davidson, C. Frondoza, D. Hungerford, Johns Hopkins University, Baltimore, MD 21239

Propagation of chondrocytes in microcarrier spinner culture has been previously shown by this laboratory to promote proliferation and maintenance of phenotypic expression, in particular type II collagen production. In order to investigate the mechanisms involved, we are developing methods of identifying genes whose expression is modified by mechanical stimulation. Some of these factors may include growth factors, cytokines, integrins, cell membrane receptors, intracellular molecules involved in signal transduction (second messengers), and transcription factors. We have created chondrocyte cDNA libraries from cells retrieved from patients with osteoarthritis which have been grown in monolayer or in spinner culture. We are optimizing the application of subtractive hybridization to these libraries to examine some of the upstream genes important in growth and phenotype maintenance in these chondrocytes. Determination of such genes is important both in gaining an understanding of the processes involved in chondrocyte growth and differentiation, and in identifying genes which may eventually be targets for gene therapy.

2745

GLUCOCORTICOID RESPONSE ELEMENT (GRE) CHARACTERIZATION IN THE OVINE β 1-ADRENERGIC RECEPTOR (β 1AR) GENE PROMOTER.

Y. T. Tseng, J. Stabila, B. McGonnigal, T. T. Nguyen and J. E. Padbury. Brown Univ. Sch. Med., Women & Infants' Hospital, Providence, RI 02905.

Unlike the β 2AR, the β 1AR gene promoter has been less well characterized. The lack of hormone responsiveness of the β 1AR in the fetus and upregulation by steroids in the newborn and adults may represent a unique transcription mechanism of this physiologically critical receptor. With transient transfection of progressively deleted ovine β 1AR gene promoter, we have identified the promoter region conferring glucocorticoid responsiveness. Gel retardation assay using this region of the promoter showed a cell type specific effect where band shifts were observed in SK-N-MC but not in C6 cell nuclear extracts. We used DNase I footprinting to confirm that nucleotides -1260 ~ -1247, upstream of a GRE 1/2 site, interact with SK-N-MC cell nuclear proteins. The wild type nucleotides and its corresponding mutant sequences were subcloned into the pGL2-C vector for further studies. While the wild type sequence showed different band shifts from that of mutant sequence in gel retardation assay, no supershift was observed when a glucocorticoid receptor antibody was included in the assay mixture. Further, transient transfection indicated a significant increase in transcriptional activity induced by the wild type sequences. We conclude that nucleotides -1260 ~ -1247, although conferring cell-type specific glucocorticoid responsiveness, do not appear to interact with GR directly. We speculate that this region in the ovine β 1AR promoter may be responsible for its unique transcription regulation. (Supported by NICHD 2P01 HD11343).

2742

nm155 BINDS TO INTRACISTERNAL A-PARTICLES PROXIMAL ENHANCER ELEMENT (IPE) AND POLYPYRIMIDINE TRACT-SPLICING FACTOR (PSF).

H. Schmitt, R. Moreland, N. Savelleva, L. Hafer and A. Traish. Boston University School of Medicine, Boston, MA 02118.

We have previously reported on the loss of expression of a novel 55-kDa nuclear protein (nm155) in estrogen receptor-negative (ER-) human breast tumors. In this study we determined: a) the binding of nm155 to unique DNA response elements by gel mobility shift assays, b) interactions of nm155 with nuclear proteins by co-immunoprecipitation and c) distribution of nm155 in human breast cancer tissues by immunocytochemistry. nm155 bound to a unique 24 base pair DNA sequence, previously reported as "intracisternal A-particles proximal enhancer element (IPE)." nm155 bound IPE specifically and with high affinity. Immunoprecipitation of MCF-7 cell lysates with antibodies to nm155 produced two distinct and specific bands of 55 and 100 kDa. Western blot analyses with antibodies specific to human PSF detected a specific 100-kDa band, suggesting that nm155 may associate with PSF. nm155 was localized only to the nucleus and the intensity of staining varied significantly among breast tumors with various pathological characteristics, suggesting a heterogeneous phenotype. The data from this study suggest that nm155 may be involved in RNA processing, transcription and may represent a novel tumor marker in human breast cancer. Supported by a grant from DOD DAMD 17-94-J-4468.

2744

TRANSCRIPTION REGULATION OF RAT HMGI-C EXPRESSION BY RAF-1/MEK/ERK SIGNALING PATHWAY X. Wen, H. Lin and D.K. Ann. Dept. of Mole. Pharmacol. and Toxicol. Univ. of South. Cal., Los Angeles, CA 90033

HMGI-C cDNA, a non-histone chromosome protein, was previously isolated as a gene upregulated following Raf-1 activation. This study is to understand the molecular mechanism underlying Raf-1-mediated HMGI-C gene expression. First, the induction of HMGI-C gene expression was indicated to occur at transcription level. HMGI-C cDNA was then used to screen rat DASHII genomic library. A clone containing 12 kb of 5' flanking region was isolated, in which more than 4kb was sequenced. Subsequently, transcription initiation sites were localized at 702 to 744 nucleotide upstream of the ATG initiation codon by 5'RACE. To identify Raf-1-dependent regulatory elements (RDRE), constructs with a series of 5' deletion of HMGI-C gene linked to CAT reporter gene were made and were transiently transfected into cells containing inducible form of Raf-1 gene. Upon Raf-1 activation, no RDRE was identified within the 6.6 kb upstream of the translation initiation site. Instead, the construct containing 0.9 kb of promoter region and 0.8 kb of intron1 was shown to have significant induction of expression by Raf-1, suggesting RDRE might locate within intron1. Members of Ets transcription factor family are possibly involved in modulation of HMGI-C expression. (Supported in part by grant DE 10742 to D.K. Ann)

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An RT-PCR-based assay system for examining fiber-type specific gene expression in skeletal muscle. E.R. Chin and R.S. Williams. UT Southwestern Medical Center, Dept. of Internal Medicine, 5323 Harry Hines Blvd. Dallas TX. 75235-8573

The purpose of this study was to develop an assay system for examining fiber-type specific gene expression in small samples of skeletal muscle using reverse-transcriptase polymerase chain reaction (RT-PCR). The entire soleus (SOL), extensor digitorum longus (EDL) and flexor brevis (FB) and a sample of white gastrocnemius (WG) (~ 10 mg) were removed from adult mice (n=6). RNA was isolated using a guanidine thiocyanate extraction method and 60 μ g (EDL, FB, WG) or 120 μ g (SOL) RNA used for cDNA synthesis. Muscle cDNAs were amplified by PCR using primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), myoglobin (Mb), parvalbumin (PV), phospholamban (PL) and skeletal alpha-actin, with volumes and number of cycles shown to be in the linear range for each primer pair. PCR products were analyzed on ethidium bromide gels using densitometry. Levels of expression are shown as ratios relative to actin (mean \pm SE; ND = not detected).

	GAPDH:Actin	Mb:Actin	PV:Actin	PL:Actin
SOL	0.95 \pm 0.11	1.90 \pm 0.16	0.05 \pm 0.01	0.26 \pm 0.05
EDL	2.05 \pm 0.45	0.67 \pm 0.11	3.08 \pm 0.83	0.09 \pm 0.04
FB	1.06 \pm 0.39	0.99 \pm 0.62	0.98 \pm 0.30	ND
WG	1.69 \pm 0.32	0.07 \pm 0.02	1.37 \pm 0.18	0.02 \pm 0.03

Thus, muscles can be categorized into highly glycolytic (GAPDH:Actin ratio > 1.5; EDL, WG) and highly oxidative (Mb:Actin ratio > 0.5; SOL, EDL, FB) as well as into fast (PV:Actin > 0.5; EDL, FB, WG) and slow (PL:Actin > 0.2; SOL) sub-types. Using these criteria for fiber-type specific gene expression, we anticipate that this RT-PCR system can be used to identify muscle fiber types at the single cell level.

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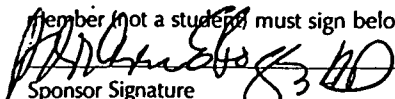
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BLACK TEA AND STEROID HORMONE RECEPTORS IN NORMAL AND NEOPLASTIC MAMMARY GLANDS IN RATS. L J Hafer, K E Murphy, A E Rogers, A M Traish, and P F Johannsen. Boston University School of Medicine & Mallory Institute of Pathology, Boston, MA.

Green tea extracts reduce chemical carcinogenesis in skin, lung, esophagus, and gastrointestinal tract of laboratory rodents. Studies of black tea extracts have yielded similar results. Mammary gland carcinogenesis in rodents is modulated by dietary factors including N-6-polyunsaturated fats (N-6 PUFA). Two studies of mammary gland carcinogenesis in rats by 7,12-dimethylbenz(a)anthracene (DMBA) showed suggestive evidence of a chemopreventive effect of green or black tea. In one (Cancer Letters, 114: 323-327, 1997), tumor multiplicity was reduced in rats given tea and a high N-6-PUFA diet. Bioassays in our laboratory have shown some evidence of reduction by black tea of DMBA-induced mammary gland tumorigenesis also in rats fed a high N-6-PUFA diet, but not in rats fed control AIN-76A diet. Tea extracts can influence estrogen metabolism and block estrogen stimulated growth of breast cancer cells(MCF-7). We examined the levels of estrogen and progesterone receptors (ER, PR) in normal and neoplastic mammary glands from rats, 16-18 weeks after DMBA, 15 or 25 mg/kg or vehicle was given by gavage. The rats were fed AIN-76A diet and drank water, 1.25% or 2.5% tea throughout the experiment. Normal glands showed suggestive evidence that PR levels were increased by tea ingestion (p=0.06) indicating that tea may have estrogenic effects. In tumors, ER and PR were markedly and statistically increased compared to normal glands, and there was a suggestive, but not statistical, reduction in ER in tumors of tea-fed rats (p=0.08). In vitro studies utilizing MCF-7 cell or human breast tumor cytosols showed that green or black tea water extracts and isolated tea components contain substances capable of binding to and activating the ER. This work was supported by grants from the USAMRMC, DAMD17-94-J-4421 and the Tea Trade Health Research Association.

Abstract Dimensions: 10.7 cm x 14.5 cm

differences were observed, compared to those resulting from exposures to the straight chain glycol ether, DiEGME. Supported by the Department of the Air Force, Contract No. F41624-96-C-9010.

1553 REDUCTION IN BRCA-1 EXPRESSION INDUCED BY BENZO[A]PYRENE IN BREAST AND OVARIAN CANCER CELLS.

D Romagnolo^{1,2}, B D Jeffy¹, E U Schultz¹, M E Ariza², T G Bowden², and M A Nelson². ¹Lab Mammary Gland Biol, Dept. Anim Sci. and ²Arizona Cancer Center, The University of Arizona, Tucson, AZ.

Loss of BRCA-1 expression due to regulatory and mutagenic events may favor neoplastic transformation. One of the environmental xenobiotics known to induce DNA damage is benzo[a]pyrene (B[a]P). We investigated the effects of acute and chronic exposure to B[a]P on expression of BRCA-1 in breast (MCF-7) and ovarian (BG-1) cancer cells. Acute exposure to B[a]P (10 μ M) for 24, 48, and 72h induced cell cycle arrest in S-phase and a concomitant decrease (2-, 8-, and 25-fold, respectively) in BRCA-1 mRNA expression, as determined by RT-PCR and densitometric analyses. Morphological analysis by cytospin and 7-amino-actinomycin-D staining followed by flow cytometry indicated that 80% of MCF-7 cells underwent apoptosis 72h after treatment with B[a]P. Acute experiments with B[a]P and its diol epoxide BPDE (benzo[a]pyrene-r-7,t-8-dihydrodiol-t-9,10-epoxide (\pm)anti) confirmed that the reduction in expression of BRCA-1 mRNA in BG-1 and MCF-7 cells was both time- and dose-dependent. Chronic exposure of MCF-7 cells to B[a]P (40 nM) for 120 days resulted in a 8-fold reduction in expression of BRCA-1 mRNA. These cumulative data indicate that both acute and chronic exposure to B[a]P may alter the expression of BRCA-1 and have important implications with regards to the role of cigarette smoking in breast cancer development. (Supported by ADCRC contract #9722, R29GA70145-01 and NIEHS06694).

1554 TCDD INDUCES THE EXPRESSION OF THE RECEPTOR TYROSINE KINASE ERBB2 IN T47D BREAST CANCER CELLS.

W G R Angus and C R Jefcoate. Department of Pharmacology and Environmental Toxicology Center, The University of Wisconsin, Madison, WI.

The receptor tyrosine kinase (RTK) ErbB2 (HER2/neu) is overexpressed in a large percentage of breast cancers. ErbB2 can heterodimerically pair with the EGF receptor (ErbB1) and two other family members, ErbB3 and ErbB4. Different signalling pathways, including the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3 kinase (PI3K), can be activated depending upon which ErbB proteins dimerize. Estrogen (E2) is reported to downregulate ErbB2. Since 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is considered an antiestrogen and downregulates the ER, we examined the possibility that TCDD could increase ErbB2 by releasing the E2-induced downregulation. TCDD, 10 nM, more than doubled the expression of ErbB2 and ErbB3 in T47D breast cancer cells by 24 hours. PCR analysis of ErbB2 and ErbB3 message also indicated an induction by 10 nM TCDD at 24 hours. These data suggest that TCDD could play an important role in the etiology of breast cancer by altering the expression of RTKs, which may lead to altered cellular signalling. Supported by grants DAMD 17-94-J-4054 (CRJ) and NRSA 1-F32-ES05733-01 (WA)

1555 CHARACTERIZATION OF THE p53-MEDIATED PATHWAY IN HUMAN MAMMARY EPITHELIAL CELLS IN RESPONSE TO IONIZING RADIATION.

K Mitro-Meyer, S M Hess, and S A Leadon. Department of Radiation Oncology and Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, NC. Sponsor: D Holbrook.

The long term objective of our research is to provide a better understanding of how breast epithelial cells respond to DNA-damaging agents. Recent studies in our lab show that cultured human mammary epithelial cells (HMEC) express wild-type p53 (exons 4-9), yet do not demonstrate a G1 arrest after treatment with 4 Gy of ionizing radiation (IR). Therefore, we investigated further the p53-mediated response of HMEC following treatment with IR, and compared these results to those obtained from matched sets of normal mammary fibroblasts. Two HMEC strains (HMEC 184 and 161) with a finite lifespan and their fibroblast (Fb) counterparts were irradiated with 4 Gy of IR or sham-irradiated, and incubated for various times from 0-16 hrs post-irradiation. After isolation of whole cell lysates, Western analyses were

performed to examine the expression of p53 and its downstream targets associated with G1 arrest, p21^{WAF1} and GADD45. Following exposure to IR, both 184 Fb and 161 Fb show a rapid induction of p53 protein, with maximal increases occurring within 3 hrs. The p21^{WAF1} protein levels also increase in these cells, with a 6-fold induction in the 161 Fb occurring by 6 hrs post-treatment, and a 3-fold induction in the 184 Fb occurring between 3-12 hrs post-treatment. In marked contrast, p53 and p21^{WAF1} in HMEC 184 and 161 are not induced over the time course examined. Furthermore, the expression of GADD45 does not increase in HMEC 161 after treatment. The protein levels of Mdm2, a negative regulator of p53, were measured following IR to determine whether the lack of p53 induction is associated with an elevated expression of Mdm2. Our data indicate that HMEC maintain higher basal levels of Mdm2 than their Fb counterparts. In addition, Mdm2 is induced by 3 hrs in both HMEC lines and mammary Fb. These data indicate that breast epithelial cells exhibit a different p53-mediated response to IR than mammary Fb and that the signaling pathway downstream of p53 is abrogated in HMEC.

* **1556** NUCLEAR EXPRESSION OF THE ARYL HYDROCARBON RECEPTOR (AHR) IN 7,12-DIMETHYLBENZ[*a*]ANTHRACENE (DMBA)-INDUCED RAT MAMMARY TUMORS AND HUMAN BREAST CANCER CELL LINES.

A F Trombino¹, R A Matulka², S Yang³, L J Hafer¹, A E Rogers^{1,3}, D H Sherr^{1,2}. ¹Departments of Pathology and ²Environmental Health, Boston University School of Medicine and Public Health, ³Mallory Institute of Pathology, Boston, MA.

The AhR is a transcription factor which upon activation by polycyclic aromatic hydrocarbons (PAH) translocates to the nucleus resulting in altered gene expression. Western analyses of nuclear extracts from normal and DMBA-induced mammary tumors determined that AhR protein was present in nuclei of malignant but not normal cells. Immunohistochemistry confirmed that AhR was present in nuclei of malignant epithelial cells. To assess whether AhR activity is a factor in human breast cancer, nuclear protein and whole cell extracts from human breast cancer cell lines were analyzed by immunoblot and RT-PCR. These data demonstrated high AhR mRNA and protein expression in malignant cell-lines (MCF-7, MBA-MB-231, CAMA-1 and HS578T), in MCF-10F pre-malignant breast cell line, and in PAH-transformed breast cell lines (184-A4 and 184-B5). Non-malignant breast epithelial cells (HS578BST and 184) expressed little or no AhR mRNA and protein. To determine if AhR activity influences pre-malignant or malignant cell growth, three AhR antagonists, α -naphthoflavone, galangin and indol-3-carbinol were added to pre-malignant MCF-10F and malignant MCF-7, MBA-MB-231 and HS578T clones. All 3 inhibitors significantly slowed MCF-10F cell growth ($p < 0.05$), while little inhibition of malignant cell growth was observed. Inhibition of MCF-10F cell growth was not mediated by apoptosis, as illustrated by propidium iodide staining. These results are consistent with data obtained in the rat system and with our hypothesis that AhR over-expression is accompanied by constitutive AhR activation. Also, it suggests that AhR plays a role in growth regulation in a pre-malignant cell line and that once cells have malignantly transformed they may be non-responsive to AhR-dependent growth signals.

1557 ESTROGENICITY OF MEDICINAL BOTANICALS IN FEMALE RATS.

C L Eagon², M S Elm^{1,2}, A G Teepe^{1,2}, H H Ayer¹, C Rich³, N B Tress¹ and P K Eagon^{1,2,3}. ¹VA Medical Center; ²University of Pittsburgh School of Medicine; ³Carlow College, Pittsburgh PA.

Introduction: Medicinal botanicals consisting of extracts from a number of plants have been used for centuries to relieve various gynecological symptoms. Further, medicinal botanicals are of increasing interest to persons seeking alternative health care and self-treatment. Women who have or are at risk for breast cancer or liver disease pose a particular problem when using such materials, since little is known about their safety, potency, and hormonal properties. In this study, we evaluated a number of these extracts, i.e., dang gui root, vitex berry, hops flower, blue and black cohosh roots, licorice, and ginseng root, for estrogenic potency using several different criteria. **Methods:** Extracts were tested in an *in vitro* competitive estrogen receptor (ER) binding assay in parallel with diethylstilbestrol and genistein. Some extracts were tested further for estrogenicity *in vivo* in female rats; extracts were added to a standard liquid rat diet and fed to ovariectomized (OV) female rats for 3 weeks. Changes in uterine weight (UW) was measured. Hepatic estrogenic

response was measured as changes in serum ceruloplasmin (CP) levels and liver steady-state CP mRNA levels. Serum LH levels were also quantitated to evaluate hypothalamic/pituitary response. **Results:** Significant dose-dependent inhibition of radiolabelled estradiol binding to ER was observed with dang gui, licorice, both cohosh types, vitex and hops extracts. Vitex, dang gui, and cohosh resulted in UW increase. Dang gui and hops elicited a significant increase in serum CP levels ($p < 0.05$ as compared to untreated OV female rats). Further, dang gui, hops, vitex and ginseng increased hepatic CP mRNA levels (2 to 4.5-fold, $p < 0.05$). LH levels were decreased significantly ($p < 0.05$) by hops (35% reduction), vitex (31% decrease) and cohosh (25% decrease), indicating estrogenicity at the hypothalamic/pituitary level as well. **Conclusions:** These studies verify that these medicinal botanicals demonstrate measurable and significant estrogenic activities in several tissues. The estrogenicity of such preparations may be clinically useful; conversely, these herbs might be avoided in conditions in which estrogens are contraindicated.

1558 MAMMARY GLAND CARCINOGENESIS, BLACK TEA AND DIETARY FAT.

A E Rogers, L J Hafer, Y S Iskander, and S Yang. *Mallory Inst. of Pathology and Boston University School of Medicine, Boston MA.*

Epidemiological studies indicate association of reduced esophageal, colorectal and pancreatic cancer risk with green tea consumption in China; studies in western populations have not yielded consistent results on effects of black tea on cancer risk. In laboratory rodents extracts of green or black tea reduce carcinogenesis by certain carcinogens in lung, gastrointestinal tract and skin and by ultraviolet light in the skin. Effects of tea have not been evaluated fully in rodent breast cancer models, but results of two experiments have suggested protection by tea in female Sprague-Dawley (S-D) rats given 7,12-dimethylbenz(a)anthracene (DMBA). Rats fed a natural product diet with 1% green tea catechins showed somewhat increased tumor latency and decreased tumor size compared to controls not fed tea (Cancer Letters 83:149, 1994); rats fed a high N-6-polyunsaturated fat (PUFA) diet that promotes mammary tumorigenesis and given 1.25% black tea extract to drink had fewer mammary fibroadenomas than rats given water but similar numbers of adenocarcinomas (Cancer Letters 114:323, 1997). To evaluate more fully the possible influence of black tea on DMBA-induced mammary gland tumors, 3 experiments (expt.) were performed comparing tumorigenesis in: (1) rats given 25 mg/kg DMBA, fed AIN 76A diet and given 1.25% or 2.5% tea extract or water to drink; (2) rats given 15 mg/kg DMBA, the same diet and drinking fluids as in (1); (3) rats fed AIN 76A or a high-N-6-PUFA diet and given 15 mg/kg DMBA and 2% tea or water to drink. The rats readily accepted tea and gained weight normally. Total tea intake in expt. 1 represented 58 ± 13 or 106 ± 22 grams of extracted tea leaf. In the 3 experiments there was no consistent effect of tea on tumorigenesis in rats fed AIN 76A diet. In Expt. 3, in rats fed the high N-6-PUFA diet and given 2% tea, tumor number and burden were statistically reduced ($p < 0.05$) compared to rats fed the high N-6-PUFA diet given water. It appears that black tea reduces mammary tumorigenesis in rats fed a high N-6-PUFA diet. This work was supported by grants from the Tea Trade Health Research Association and USAMRMC, DAMD 17-94-J-4221.

1559 BLACK TEA AND STEROID HORMONE RECEPTORS IN NORMAL AND NEOPLASTIC MAMMARY GLANDS IN RATS.

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Green tea extracts reduce chemical carcinogenesis in skin, lung, esophagus, and gastrointestinal tract of laboratory rodents. Studies of black tea extracts have yielded similar results. Mammary gland carcinogenesis in rodents is modulated by dietary factors including N-6-polyunsaturated fats (N-6 PUFA). Two studies of mammary gland carcinogenesis in rats by 7,12-dimethylbenz(a)anthracene (DMBA) showed suggestive evidence of a chemopreventive effect of green or black tea. In one (Cancer Letters, 114: 323-327, 1997), tumor multiplicity was reduced in rats given tea and a high N-6-PUFA diet. Bioassays in our laboratory have shown some evidence of reduction by black tea of DMBA-induced mammary gland tumorigenesis also in rats fed a high N-6-PUFA diet, but not in rats fed control AIN-76A diet. Tea extracts can influence estrogen metabolism and block estrogen stimulated growth of breast cancer cells (MCF-7). We examined the levels of estrogen and progesterone receptors (ER, PR) in normal and neoplastic mammary glands from rats,

16-18 weeks after DMBA, 15 or 25 mg/kg or vehicle was given by gavage. The rats were fed AIN-76A diet and drank water, 1.25% or 2.5% tea throughout the experiment. Normal glands showed suggestive evidence that PR levels were increased by tea ingestion ($p = 0.06$) indicating that tea may have estrogenic effects. In tumors, ER and PR were markedly and statistically increased compared to normal glands, and there was a suggestive, but not statistical, reduction in ER in tumors of tea-fed rats ($p = 0.08$). In vitro studies utilizing MCF-7 cell or human breast tumor cytosols showed that green or black tea water extracts and isolated tea components contain substances capable of binding to and activating the ER. This work was supported by grants from the USAMRMC, DAMD17-94-J-4421 and the Tea Trade Health Research Association.

1560 VITAMIN D₃ AND GENISTEIN INDUCE DETOXIFICATION ENZYMES IN MCF-7 HUMAN BREAST CANCER CELLS.

A H Pakh, and M J DeLong. *Department of Environmental and Occupational Health, Emory University School of Public Health, Atlanta, GA.*

Two dietary compounds, vitamin D₃ and genistein, inhibit cell proliferation and induce apoptosis in vitro. The most active metabolite of vitamin D₃, 1,25(OH)₂D₃, is associated with chemoprotective effects by inhibiting cell growth and enhancing apoptosis in mammary cell lines with and without estrogen receptors. Genistein, a naturally occurring isoflavonoid phytoestrogen, has been related to inhibition of mammary cancer by preventing angiogenesis and is found in citrus fruits and soy products. The aim of this study was to determine the relationship of vitamin D₃ and genistein, independently, to the induction of two phase II detoxification enzymes, glutathione S-transferase (GST) and NAD(P)H: quinone reductase (QR), in MCF-7 human breast cancer cells as a possible mechanism to prevent carcinogenesis. The MCF-7 human breast cancer cell line is positive for estrogen and vitamin D receptors and is associated with a chemoprotective response to both vitamin D₃ and genistein. Cells were treated with a single dose of 100nM vitamin D₃ or 10 uM and 25 uM genistein with ethanol as the vehicle for 2, 3, and 4 days. Cells were assayed for levels of QR and GST spectrophotometrically. Vitamin D₃ elevated both QR and GST levels on all days with statistically significant QR (1.36 fold) and GST (1.43 fold) elevations for day 2 ($p = 0.007$ and $p = 0.004$, respectively). QR and GST enzyme elevations were also significant with 25uM genistein for day 2 with a 1.23 ($p = 0.001$) and 1.22 ($p = 0.05$) fold increase, respectively. QR and GST were also elevated at 10uM and later days though not with strong significance. This study along with the existing literature provides evidence that vitamin D₃ and genistein protect against mammary carcinogenesis not only by regulating cell growth in mammary cancer cells but also by inducing QR and GST levels.

1561 A NEPHROTOXIC METABOLITE OF HYDROQUINONE INDUCES RENAL CELL TUMORIGENESIS IN EKER RATS.

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The Eker rat carries a germline mutation in the Tsc-2 tumor suppressor gene which predisposes these rats to the development of renal cell carcinoma (RCC). Eker rats have been utilized as a model to study the influence of genetic susceptibility in chemically-induced nephrocarcinogenesis. Hydroquinone (HQ) is a rat nephrocarcinogen that generally tests negative in standard mutagenicity assays. 2,3,5-tris-(glutathion-S-yl)HQ (tris-(GSyl)-HQ) is a potent nephrotoxic metabolite of HQ. To determine the effect of tris-(GSyl)HQ on renal tumorigenesis, Eker rats were administered tris-(GSyl)HQ from 2 to 12 months of age (2.5 µmol/kg, i.p., 5 days/week for 4 months; 3.5 µmol/kg 5 days/week for 6 months). Tumor multiplicity and incidence increased 3-fold in treated rats. Adenoma multiplicity increased from 11/kidney in controls to 34/kidney in tris-(GSyl)HQ treated animals. The incidence of RCCs increased from 14% to 44%. The ratio of RCC to renal cell adenoma was identical in control (0.026) and treated (0.025) Eker rats, suggesting that tris-(GSyl)HQ acts at the initiation stage, rather than by promoting the growth of existing lesions. tris-(GSyl)HQ-treated rats had numerous toxic tubular dysplasias of a form rarely present in aging Eker rats. These lesions were found as early as 4 months following initiation of treatment. In 12 month old animals, adenomas were found arising within these lesions. These "preneoplastic" lesions are believed to represent "early transformation" within tubules undergoing regeneration in response to toxic injury. The majority of the renal cell tumors colocalize with the region of tris-(GSyl)HQ-induced acute renal injury, suggesting that HQ-mediated

Thus, contact or close proximity to the preB cell microenvironment appears necessary for AhR-dependent DMBA-induced preB cell apoptosis. Supported by NIH R01-ES06086 and Superfund Basic Research Grant #1P42ES 07381

*** 1238 MODULATION OF AROMATIC HYDROCARBON RECEPTOR EXPRESSION IN 7,12-DIMETHYLBENZ[α]ANTHRACENE-INDUCED RAT MAMMARY TUMORS.**

A F Trombino¹, S Yang¹, L J Hafer¹, A N Qadri², A E Rogers¹, D H Sherr^{1,2}. ¹Departments of Pathology and ²Environmental Health, Boston University School of Medicine and Public Health, ³Mallory Institute of Pathology, Boston, MA.

Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants implicated in the increased incidence of breast cancer. The biologic effects of PAH are likely mediated by the aromatic hydrocarbon receptor (AhR)/transcription factor. Ligated AhR translocates to the nucleus inducing a battery of genes including CYP1A1. In addition, AhR may act upon estrogen receptor and NF- κ B activity. Using a well-established rat model of DMBA-induced mammary tumorigenesis we observed AhR expression by immunohistochemistry, RT-PCR and western immunoblotting. Immunohistochemical analyses of rat tissue indicate significant AhR expression in normal breast tissue where AhR appears to be localized primarily to the cytoplasm of myoepithelial cells adjacent to ductal epithelial cells. However, in adenocarcinomas induced approximately 15 weeks after DMBA administration, AhR staining was increased with strong staining in both stromal and malignant epithelial elements. AhR mRNA levels were dramatically increased in DMBA-induced rat tumors as compared with normal tissue as assessed by quantitative RT-PCR. AhR mRNA levels in tumor samples exceeded those in normal rat liver or spleen, tissues which contain high levels of AhR mRNA. These data suggest that AhR mRNA and protein expression changes dramatically during malignant transformation. Additional studies evaluated the possibility that the AhR is constitutively activated in mammary carcinomas. Supported by NIH R01-ES06086, Superfund Basic Research Grant #1P42ES and a Veteran's Administration Medical Research Division Center Grant.

1239 KEY ROLE OF [Ca²⁺]_i IN REACTIVE OXYGEN SPECIES AND IL-1 α PRODUCTION BY TRIBUTYLTIN IN A MURINE KERATINOCYTE CELL LINE.

M Marinovich, B Viviani, C L Galli, and E Corsini. University of Milan, Institute of Pharmacological Sciences, Lab. of Toxicology, Milano, Italy.

Tributyltin (TBT) salts are potent skin irritants both in humans and rodents. Data in the literature indicates mitochondria as target of TBT effects. Here, we investigate the early intracellular molecular events that follow TBT treatment and mitochondrial impairment and the relevance of these organelles in gene-regulatory signaling pathways. Confluent HEL30 cells were treated with increasing doses of TBT (0–5 μ M). At different time thereafter, the level of intracellular Ca²⁺, the cellular oxidative activity, nuclear factor- κ B (NF- κ B) activation and IL-1 α production were measured. TBT induced a dose-related increase of intracellular Ca²⁺, that reached the plateau 4 min following treatment. The increase of intracellular Ca²⁺ was followed by an increase in cellular oxidative activity (15 min), that preceded NF- κ B activation (30 min) and IL-1 α production (4 h). All these events can be almost completely abrogated by BAPTA, an intracellular Ca²⁺ chelator. Furthermore, the partial modulation of reactive oxygen species induced by TBT observed with rotenone, an inhibitor of the electron entry from complex I to ubiquinone, or after prolonged treatment with ethidium bromide, an inhibitor of mitochondrial DNA and RNA synthesis, indicates mitochondria as the main intracellular source of reactive oxygen species. These findings confirm the pivotal role of mitochondria as the source of second messenger molecules essential for TBT-induced NF- κ B activation and IL-1 α production and indicate the rise in intracellular Ca²⁺ as the starting event.

Acknowledgments: sponsored by U. E. grant (EV5V-CT94-0508).

1240 PHENOLIC ANTIOXIDANTS, BHA AND BHQ, ACTIVATE MITOGEN-ACTIVATED PROTEIN KINASES VIA DIFFERENTIAL OXIDATIVE PATHWAYS.

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BHA and BHQ, termed phenolic antioxidants due to their chain-breaking

action during autooxidation of lipid peroxidation, are primarily used as food preservatives. Further studies showed that these phenolic antioxidants exert anti-tumorigenic effects in some experimental animal models of cancer. On the other hand, these compounds are also found to exhibit carcinogenic activity, presumably through the formation of oxidized metabolites and reactive oxygen species during redox cycling. To better understand the molecular mechanisms underlying these biological effects, we investigated the activation of mitogen-activation protein kinases (MAPK) by BHA and BHQ. On treatment of HeLa cells with BHA, c-jun N-terminal kinase 1 (JNK1) and extracellular signal-regulated protein kinase 2 (ERK2) were strongly activated in a dose- and time-dependent fashion. Similarly, BHQ also potently activated ERK2, but stimulated JNK1 activity to much lesser extent as compared with BHA. Pretreatment with free radical scavengers, N-acetyl-L-cysteine (NAC) and vitamin E, substantially inhibited ERK2 and JNK1 activation by BHA and BHQ. Inhibition of catalase with aminotriazole enhanced the effects of BHA and BHQ on induction of ERK2 and JNK1. Furthermore, both BHA and BHQ caused lipid peroxidation as evidenced by oxidation of cis-parinaric acid. However, flow-cytometry experiment showed that the treatment of BHQ leads to generation of reactive oxygen species (ROS), whereas BHA reduced the total amount of pro-oxidants. These findings suggest that ROS participate in the induction of phenolic antioxidant-elicited MAP kinase signaling pathway.

1241 INTERACTIONS BETWEEN GLUTATHIONE, OXIDATIVE STRESS, AND BENZO(A)PYRENE IN THE REGULATION OF c-Ha-RAS GENE EXPRESSION.

J K Kerzee and K S Ramos. Faculty of Toxicology and Dept. of Veterinary Physiol. & Pharmacol., Texas A&M University, College Station, TX.

Benzo(a)pyrene (BaP) and related aromatic hydrocarbons have been implicated in the formation of vascular atherosclerotic lesions in laboratory animals. Recent studies in this laboratory have identified c-Ha-ras as a critical molecular target in the deregulation of vascular smooth muscle cell (SMC) proliferation and differentiation by BaP. The ability of BaP to influence patterns of gene expression may involve induction of oxidative stress. Therefore, the present studies were conducted to examine the impact of redox status on SMC responses to BaP. Our experimental approach involved assessment of glutathione (GSH) homeostasis upon *in vitro* exposure of vascular (aortic) SMCs to BaP (3 μ M) alone or in combination with N-acetyl cysteine (NAC) (0.5 mM) or buthionine-S-sulfoximine (BSO) (50 μ M). Subcultured SMCs were growth-arrested for 72 hr and then incubated with NAC or BSO in the presence of serum for 16 hr to enhance or deplete GSH levels before BaP challenge, respectively. Exposure to BaP for 24 hr was associated with a 41% depletion of cellular GSH levels. Pre-treatment of cells with NAC afforded complete protection from BaP-induced depletion of GSH. In contrast, pre-treatment with BSO reversed the BaP effect and, in fact, resulted in marked upregulation of cellular GSH levels. Based on these results we propose that depletion of GSH by BaP induces oxidative stress which in turn may mediate its ability to influence patterns of growth-related gene expression. (Supported by NIH grant ES 04849).

1242 INTERACTION OF v-MAF WITH THE ANTIOXIDANT RESPONSE ELEMENT.

M M Halleck, T Nguyen and C B Pickett. Schering-Plough Research Institute, Kenilworth, NJ. Sponsor: I Y Rosenblum.

The purpose of this work was to determine if the oncogene v-Maf binds to the antioxidant response element (ARE). The ARE is a transcriptional enhancer whose core DNA sequence (5'GTGACNNGCA3') mediates induction of glutathione-S-transferase (GST) and NAD(P)H:quinone oxidoreductase (QR) following exposure of cells to phenolic antioxidants and redox cycling compounds. The Maf response element (MARE), an empirically derived DNA sequence (5'TGCTGACTCAGCA3') binds v-Maf, a member of the basic leucine zipper family of transcription factors. The sequence similarity between the ARE and the MARE (underlined above) suggests v-Maf could be an ARE binding protein. Gel shift analyses were used to monitor the interaction of v-Maf with the rat GST ARE and/or the rat QR ARE. The following results were obtained. 1) v-Maf, translated *in vitro* alone or in combination with Fos or Jun, did not bind to the ARE with high affinity. 2) v-Maf, transiently expressed in Hep G2 cells, bound MARE but not ARE sequences. 3) ARE binding complexes, endogenous to Hep G2 cells, were not supershifted by a panel of v-Maf polyclonal antibodies. Taken together these results demonstrate that v-Maf is not an ARE binding protein in Hep



ABSTRACT FORM

Fifth Annual Student Achievement Day

Boston University School of Medicine

April 7, 1999 * Hiebert Lounge * 9 a.m. - 5 p.m.

Deadline for submission: March 19, 1999

ESTROGEN AND PROGESTERONE RECEPTORS IN NORMAL AND MALIGNANT MAMMARY GLANDS IN TEA AND HIGH FAT DIET-FED FEMALE SPRAGUE-DAWLEY RATS. L.J. Hafer, A.M. Traish and A.E. Rogers, Advisor: A.E. Rogers, Departments of Pathology and Laboratory Medicine and Biochemistry, Boston University School of Medicine and The Mallory Institute of Pathology. Three tumorigenesis bioassays were performed in female Sprague-Dawley rats and showed no change in tumorigenesis with black tea ingestion 18 weeks post-7,12-dimethylbenz(a)anthracene (DMBA). However, a significant decrease in tumorigenesis with black tea in rats fed a high fat diet (20% corn oil) compared to rats fed a high fat diet alone was seen. Levels of estrogen and progesterone receptors (ER, PR) were measured by receptor-ligand binding assay for all experiments to determine if ingestion of black tea extracts altered the concentration of ER and PR in normal and malignant mammary glands. Black tea tended to decrease ER in normal mammary glands and tumors in all the experiments, but the decreases were not statistically significant. PR tended to be decreased in both normal glands and tumors, but the decrease was not consistently statistically significant. The high fat diet did not affect either receptor in normal mammary glands; however, it significantly decreased PR in tumors. When the high fat diet was combined with tea ingestion, there was a non-significant decrease in both receptors compared to high fat diet alone. Both ER and PR were also significantly increased in tumors compared to normals regardless of treatment. A short-term study in this same rat model was also performed to examine changes from 6 hrs. to 9 wks. after administration of DMBA. There was no effect of tea, high fat diet or age on ER and PR up to 9 wks. after DMBA administration. Uteri from these same rats showed no effect of DMBA, tea, diet or age on ER and PR. In virtually all groups, there was a non-significant decrease in ER and a concomitant increase in PR with age. Data from immunohistochemical studies further support these results. *In vitro* studies showed that green and black tea extracts phosphorylate ER in MCF-7 cells; studies are underway to extend and confirm this result. This work was supported by grants from the USAMRMC, DAMD17-94-J-4421 and the Tea Trade Health Association.

Full Name (presenting author), Department, and Advisor

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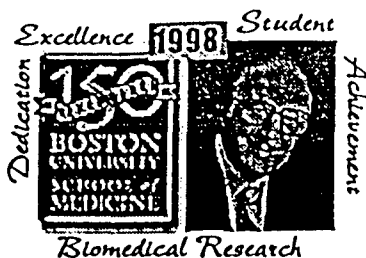
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1. Use upper case letters for the title. Underline name of principal author and continue on the same line with Advisor and Department affiliation(s). Ex. CLONING AND SEQUENCING OF THE RAT α -6 SUBUNIT GENE FOR THE GABA_A/BENZODIAZEPINE RECEPTOR (GABRA6). P.J. McLean, S.J. Russek and D.H. Farb, Advisor: D.H. Farb, Department of Pharmacology, Boston University School of Medicine.
2. Text should be single-spaced. Smallest recommended type size: 10 point.



ABSTRACT FORM

Fourth Annual Student Achievement Day

Boston University School of Medicine

March 20, 1998 * Hiebert Lounge * 9 a.m. - 5 p.m.

Deadline for submission: February 20, 1998

BLACK TEA, HIGH DIETARY FAT AND STEROID HORMONE RECEPTORS IN MAMMARY GLAND CARCINOGENESIS IN SPRAGUE-DAWLEY RATS. L.J. Hafer, K.E. Murphy, A.E. Rogers, A.M. Traish, Y.S. Iskander, S. Yang and P.F. Johansen, Advisor: A.E. Rogers, Department of Pathology and Laboratory Medicine, Boston University School of Medicine & Mallory Institute of Pathology.

Green and black tea extracts reduce chemical carcinogenesis in skin, lung, esophagus, and gastrointestinal tract of laboratory rodents. Two studies of mammary gland carcinogenesis in rats by 7,12-dimethylbenz(a)anthracene (DMBA) showed suggestive evidence of a chemopreventive effect of green or black tea. Mammary gland carcinogenesis in rodents is modulated also by several dietary factors including N-6-polyunsaturated fats (N-6-PUFA). In one (Cancer Letters, 114: 323-327, 1997), tumor multiplicity was reduced in rats given tea and a high N-6-PUFA diet. Bioassays in our laboratory have shown some evidence of reduction by black tea of DMBA-induced mammary gland tumorigenesis also in rats fed a high N-6-PUFA diet, but not in rats fed control AIN-76A diet. There was a statistical decrease in tumor burden in the tea-drinking rats fed a high fat diet ($p=0.002$) and a non-significant decrease in cumulative probability of bearing a palpable tumor compared to rats drinking deionized water and fed the same diet. Additionally, tea extracts can influence estrogen metabolism and block estrogen-stimulated growth of a breast cancer cell line (MCF-7). Therefore, we examined the levels of estrogen and progesterone receptors (ER, PR) in normal and neoplastic mammary glands from rats, 16-18 weeks after DMBA, 15 or 25 mg/kg, or vehicle was given by gavage. The rats were fed AIN-76A diet and drank water or 1.25% or 2.5% tea throughout the experiment. Normal glands from both DMBA-treated and vehicle control rats showed suggestive evidence that PR levels were increased by tea ingestion ($p=0.06$), indicating that tea may have had an estrogenic effect. In tumors, ER and PR were markedly and statistically increased compared to normal glands, and there was a non-statistical reduction in ER in tumors of tea-fed rats ($p=0.08$), suggesting that tea components might be bound to the ER. In vitro studies utilizing MCF-7 or human breast tumor cytosols showed that green or black tea water extracts and semi-purified tea components bound to and activated the ER. These findings suggest that tea extracts may influence mammary gland carcinogenesis in rats fed a high fat diet via endocrine growth control mechanisms. This work was supported by grants from the USAMRMC, DAMD17-94-J-4421 and the Tea Trade Health Research Association.

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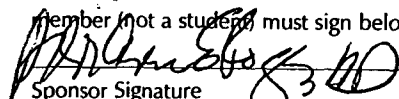
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1. Black Tea 2. Mammary Glands 3. Steroid Hormone Receptors

BLACK TEA AND STEROID HORMONE RECEPTORS IN NORMAL AND NEOPLASTIC MAMMARY GLANDS IN RATS. L J Hafer, K E Murphy, A E Rogers, A M Traish, and P F Johannsen. Boston University School of Medicine & Mallory Institute of Pathology, Boston, MA.

Green tea extracts reduce chemical carcinogenesis in skin, lung, esophagus, and gastrointestinal tract of laboratory rodents. Studies of black tea extracts have yielded similar results. Mammary gland carcinogenesis in rodents is modulated by dietary factors including N-6-polyunsaturated fats (N-6 PUFA). Two studies of mammary gland carcinogenesis in rats by 7,12-dimethylbenz(a)anthracene (DMBA) showed suggestive evidence of a chemopreventive effect of green or black tea. In one (Cancer Letters, 114: 323-327, 1997), tumor multiplicity was reduced in rats given tea and a high N-6-PUFA diet. Bioassays in our laboratory have shown some evidence of reduction by black tea of DMBA-induced mammary gland tumorigenesis also in rats fed a high N-6-PUFA diet, but not in rats fed control AIN-76A diet. Tea extracts can influence estrogen metabolism and block estrogen stimulated growth of breast cancer cells (MCF-7). We examined the levels of estrogen and progesterone receptors (ER, PR) in normal and neoplastic mammary glands from rats, 16-18 weeks after DMBA, 15 or 25 mg/kg or vehicle was given by gavage. The rats were fed AIN-76A diet and drank water, 1.25% or 2.5% tea throughout the experiment. Normal glands showed suggestive evidence that PR levels were increased by tea ingestion ($p=0.06$) indicating that tea may have estrogenic effects. In tumors, ER and PR were markedly and statistically increased compared to normal glands, and there was a suggestive, but not statistical, reduction in ER in tumors of tea-fed rats ($p=0.08$). In vitro studies utilizing MCF-7 cell or human breast tumor cytosols showed that green or black tea water extracts and isolated tea components contain substances capable of binding to and activating the ER. This work was supported by grants from the USAMRMC, DAMD17-94-J-4421 and the Tea Trade Health Research Association.

Abstract Dimensions: 10.7 cm x 14.5 cm

**BLACK TEA AND ESTROGEN AND
PROGESTERONE RECEPTORS IN
THE RAT MAMMARY GLAND**

**Laurie J. Hafer, Kristine E. Murphy,
Adrianne E. Rogers, MD & Abdulmaged M. Traish, PhD**

**Boston University School of Medicine
& Mallory Institute of Pathology**

Evidence of anticarcinogenic effects of dietary components has been reported. Extracts of green tea in particular have been shown to prevent or reduce carcinogenicity in the skin, lung, esophagus, forestomach and duodenum of laboratory rodents. Studies of black tea extracts have yielded similar results, but have been less extensive. Breast cancer, a leading cause of morbidity and mortality among women, has been shown to be modulated also by dietary intake in animal models, particularly intake of unsaturated fats, Vitamin A and selenium. Three studies of mammary gland carcinogenesis, in mice or rats, showed minimal or no evidence of a chemopreventive effect of tea. Bioassays in our laboratories have provided some evidence that tea reduced carcinogenesis in rats fed a high fat diet, but not in rats fed a control diet.

Since mammary glands and tumors are endocrine responsive and require estrogen for growth, we examined the levels of estrogen and progesterone receptors (ER, PR) in normal mammary glands and mammary gland tumors in the first bioassay by receptor-ligand binding assays. We have found suggestive evidence that PR levels were greater in normal mammary glands in rats given tea than in rats given water ($p=0.06$). Since PR synthesis is under estrogen control, we postulated that tea contains substances capable of binding the ER and may modulate its activity. Additional supporting evidence for this hypothesis was a suggestive, but not statistical, reduction in ER in tumors of tea-fed rats ($p=0.08$), indicating some tea components might be bound to the ER. To test this

**Keywords: Estrogen Receptor, Progesterone Receptor, Steroid Hormone
Receptors, Breast Cancer, Phytoestrogens**

This work was supported by the U.S. Army Medical Research and Material Command under DAMD17-94-J-4421 and a grant from the Tea Trade Health Research Association.

premise, we carried out preliminary experiments using tea extracts. In the cytosols of two ER(+) human breast cancers, two tea extracts, containing primarily catechins or theaflavins, were shown to markedly inhibit [³H]-estradiol binding to ER. Similar results, using six different tea extracts and calf uterus as a source of ER, were shown. Preliminary in vitro experiments, using MCF-7 cells grown in RDGGS media at 37°C were performed. Cells were exposed to estradiol and tea extracts, lysed in buffer containing phosphatase inhibitors and analyzed by electrophoresis and immunoblotting, using our site-directed monoclonal antibody, EVG-F9. This antibody can distinguish between activated and non-activated forms of ER based on their electrophoretic mobility. A preliminary gel and blot showed that tea extracts do phosphorylate ER like estradiol does. These in vivo and vitro observations support the view that tea contains estrogen-like substances that can activate the ER. Therefore, these substances may influence mammary gland carcinogenesis, particularly in rats fed a high fat diet, via endocrine mechanisms.

Work is ongoing to determine whether ingestion of black tea extracts alters steroid hormone receptor concentration, distribution and activation in mammary glands or tumors, in rats fed control or high fat diets and studied at intervals from hours to months after DMBA administration; to correlate the results of biochemical and immunohistochemical receptor analyses to evaluate the hypothesis that tea and dietary fat influence mammary gland tumorigenesis via endocrine mechanisms; and to identify active tea extracts and compare their estrogenic and anti-estrogenic activities as defined by in vivo and in vitro biochemical, morphological and functional endpoints.

Histopathobiology of Neoplasia

"Black Tea and Mammary Carcinogenesis in Rats", Laurie J. Hafer, Yvette S. Iskander, Sylvia Marecki, Adrienne E. Rogers, MD, Boston University School of Medicine and Mallory Institute of Pathology, Department of Pathology and Laboratory Medicine

Evidence of anticarcinogenic effects of diet components has been reported. Extracts of green tea in particular have been shown to prevent or reduce carcinogenicity in the skin, lung, esophagus, forestomach and duodenum of laboratory rodents. Studies of black tea extracts have yielded similar results, but have been less extensive. Breast cancer, a leading cause of morbidity and mortality among women, has also been shown to be susceptible to dietary intake in animal models, particularly intake of unsaturated fats, Vitamin A and selenium. Thus, the question of the effect of tea on mammary carcinogenesis was posed. Since the majority of the tea drinkers in the west consume black tea, the decision was made to examine its effect in rats. Female Sprague-Dawley rats were randomized into either deionized water or tea drinking groups and fed an AIN-76A diet. At 55 days of age, the rats were given a single dose of 7,12-dimethylbenz(a)anthracene (DMBA) by gastric gavage. Rats were palpated twice weekly for tumors and killed by CO₂ inhalation when the tumors reached 3 cm in diameter. The remaining animals were killed 16 weeks post-DMBA administration. Tumor latencies, incidences and numbers were determined for each of the groups. Two experiments were performed using identical protocols except the dose of DMBA which was 25 mg/kg (Expt #1) or 15 mg/kg (Expt #2). Rats were given 1.25% or 2.5% tea extract. There was no statistically significant effect of black tea on mammary tumor development in either experiment. Ongoing studies are examining the morphological, biochemical, and molecular changes in tissue samples obtained from these animals.

Results:

	Tumor Latency (d)	Tumor Incidence (%)	Tumor Number per TBAA
Expt#1:			
Control	80	60	2.9±1.7
1.25% Tea	71	75	3.4±2.3
Control	76	62	2.8±1.9
2.5% Tea	72	68	3.2±2.1
Expt#2:			
Control	101	62	2.0±1.2
1.25% Tea	90	40	1.8±1.2
Control	103	45	1.7±0.8
2.5% Tea	98	40	2.0±1.6

^aTBA=Tumor bearing animal

This research is supported by the Tea Trade Health Research Association and USAMRDC Breast Cancer Research Training Grant.

The Inhibitory Effects of Transforming Growth Factor- β 1 on Breast Cancer Cell
Proliferation are Mediated Through Regulation of Aberrant NF- κ B/Rel Expression

Mika A. Sovak¹, Marcello Arsura², Gregory Zanięski², Kathryn T. Kavanagh¹, and Gail E.
Sonenshein^{2*}

Departments of Pathology and Laboratory Medicine¹ and Biochemistry², and Program in
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Running title: Growth inhibition by TGF- β 1 mediated via NF- κ B/Rel

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NF- κ B/Rel transcription factors normally exist in non-B cells, such as epithelial cells, in inactive forms sequestered in the cytoplasm with specific inhibitory proteins, termed I κ Bs. Recently, however, we discovered that breast cancer is typified by aberrant constitutive expression of NF- κ B/Rel factors. Since these factors control genes that regulate cell proliferation, here we analyzed the potential role of NF- κ B/Rel in the ability of TGF- β 1 to inhibit growth of breast cancer cells. Decreased growth of Hs578T and MCF7 breast cancer cell lines upon TGF- β 1 treatment correlated with a drop in NF- κ B/Rel binding. This decrease was due to a decrease in phosphorylation, and resulting stabilization of the inhibitory protein I κ B- α . Ectopic expression of c-Rel in Hs578T cells led to maintenance of NF- κ B/Rel binding and resistance to TGF- β 1-mediated inhibition of proliferation. Thus, inhibition of the aberrantly activated, constitutive NF- κ B/Rel plays an important role in arrest of proliferation of breast cancer cells, suggesting NF- κ B/Rel may be a useful target in the treatment of breast cancer.

Differential Expression and Distinct Functions of Interferon Consensus Binding Protein and Interferon Regulatory Factor 4 in Macrophages¹

Sylvia Marecki*, Michael L. Atchison[†], and Matthew J. Fenton*²

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¹This work was supported by National Institutes of Health grant GM57053 (MJF), and Boston University Graduate Research Student Fellowship (SM).

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KEYWORDS: monocytes/macrophages, gene regulation, transcription factors

**Regulation of Interferon Consensus Sequence Binding Protein (ICSBP)
Expression in Murine Macrophages¹**

Wanee Kantakamalakul^{*2}, Alexander D. Politis^{*2}, Sylvia Marecki[†], Teri Sullivan^{*},
Keiko Ozato[‡], Matthew J. Fenton[†], and Stefanie N. Vogel^{*3}

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of Health, Bethesda, MD 20892

Keywords: monocytes/macrophages; lipopolysaccharide; interferon



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ABSTRACTS

INVOLVEMENT OF PHOSPHOLIPASE D IN LIPOPOLYSACCHARIDE-INDUCED NF- κ B ACTIVATION IN MOUSE MACROPHAGE-LIKE J774.1 CELLS

M. Nishijima, H. Yamamoto, and K. Hanada
Dept of Biochem. & Cell Biol., National Institute of Infectious Diseases, Tokyo, Japan

We have previously reported that exposure of J774.1 cells to lipopolysaccharide (LPS) induces rapid production of the cellular diacylglycerol (DAG) from phosphatidylcholine (PC). Here we examined what type of phospholipase mediated the DAG production from PC. For this, the phosphorous moiety of PC among various phospholipids was specifically labelled by incubating J774.1 cells with [32 P]lysophosphatidylcholine. Exposure of the labelled cells to LPS induced the rapid formation of [32 P]phosphatidyl butanol (PBut) in the presence of 1% butanol and the increase of [32 P]phosphatidic acid level in the presence of 200 μ M propranolol, an inhibitor of phosphatidate phosphohydrolase (PAP). Butanol and propranolol inhibited not only the increase of DAG level but also NF- κ B activation in response to LPS. Even in the presence of propranolol or butanol, LPS-induced NF- κ B activation was rescued when cellular DAG level was increased by pretreatment with bacterial PC-PLC. These results indicated that LPS stimulus activates PLD toward PC, resulting in the formation of PA which was then converted to DAG by PAP, and that the DAG production from PC by the PLD/PAP pathway is upstream of the NF- κ B activation in response to LPS in J774.1 cells.

RhoA act's κ B

H-7 & calphostin C = PKC inh.
Staur. blocks TPA & PMA

Protein Kinase CK2 Activation is Mediated by the MAP Kinase Pathway in LPS-stimulated Macrophages.

Matthew J. Fenton, Tracey A. Lodie, Terry K. Means, Sylvia Marecki, and Dominic Roca. The Pulmonary Center, Boston University School of Medicine, Boston, MA 02118.

Stimulation of macrophages by Gram-negative lipopoly-saccharide (LPS) rapidly leads to the activation of several protein kinases, including the MAP kinases (MAPK). We previously reported that LPS stimulation also up-regulates the enzymatic activity of protein kinase CK2, although the signaling cascade that leads to CK2 activation is unknown. We performed additional studies in order to determine if CK2 activation was a consequence of MAPK activation. Our studies revealed that CK2 activity was rapidly and transiently up-regulated in LPS-stimulated RAW264.7 murine macrophages. We found that PD98059, an inhibitor of Erk kinase activation by the MAPK kinase MEK-1, blocked LPS-induced up-regulation of CK2 activity. In contrast, the p38 kinase inhibitor SB203580 did not block CK2 activation by LPS. This finding suggests that CK2 activation was mediated by the Erk kinase signaling cascade, but not by the p38 kinase cascade. We also found that PD98059 blocked LPS-induced serine phosphorylation of PU.1, an Ets-like transcription factor that is a substrate for CK2. In transient transfection analyses, the MEK inhibitor selectively blocked LPS-inducible activation of the PU.1-dependent CD11b promoter. These studies demonstrate that activation of CK2 and PU.1, as well as PU.1-dependent genes, appears to require the Erk signaling cascade in LPS-stimulated macrophages.

Jiahui Han Scripps

LPS - intra. signaling

looks like $IKB\alpha$ but not β activated by LPS.

TXY = MEK (MAPKINASE)

TEY = ERK \rightarrow ERK-1, myc, NFIL

TGY p38 \rightarrow ERK-1, CHOP10

TPY = JNK \rightarrow ATF2, ERK-1



Macrophage Biology

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429 Interferon Consensus Sequence Binding Protein (ICSBP) Can Mediate Both Transcriptional Activation and Repression in Macrophages

Sylvia Marecki and Matthew J. Fenton Pulmonary Center and Department of Pathology, Boston University Medical Center, Boston, MA 02118, USA.

Interferon consensus binding protein (ICSBP) is a member of the growing family of interferon regulatory factors (IRF). ICSBP is expressed in both lymphoid and myeloid cells, and has been reported to repress the expression of several interferon-responsive genes. ICSBP and the related protein interferon regulated factor-4 (IRF-4, Pip, LSIRF), can bind specifically to the Ets-like protein PU.1 and to composite IRF/PU.1 sites. Both proteins are required for immunoglobulin light chain enhancer function in B cells, whereas the function of ICSBP/PU.1 complexes in other immune cells has not been previously examined. We measured the capacity of ICSBP to regulate the function of distinct promoters in RAW264.7 murine macrophages. Here we show that ICSBP/PU.1 complexes can mediate transcriptional activation via IRF/PU.1 composite sites in macrophages. Furthermore, ICSBP can function as a transcriptional activator and as a transcriptional repressor of different promoters within the same cell. We found that ICSBP could activate transcription of both the macrophage scavenger receptor and CD11b promoters, whereas ICSBP suppressed transcription of the IL-1 β and H-2L^d MHC class I promoters. Furthermore, activation of the scavenger receptor and CD11b promoters by ICSBP did not depend on the presence of PU.1. This suggests that ICSBP can activate transcription independent of PU.1, but can also function in conjunction with PU.1 to activate transcription of promoters containing composite IRF/PU.1 sites. Together, these data suggest a complex molecular mechanism that regulates the expression of interferon-responsive genes by ICSBP in macrophages.

431 Bone marrow cells cultured *in vitro* become susceptible to infection with porcine reproductive and respiratory syndrome virus.

Janneke Meulenberg, Jan Castrop, John Voermans, Janneke Samson, Bob Reus, and André Bianchi. Department of mammalian virology, Institute for Animal Science and Health, NL-8200 AB The Netherlands.

Porcine reproductive and respiratory syndrome virus (PRRSV) has a very restricted host specificity. It infects preferentially porcine alveolar lung macrophages, macrophages of other tissues, and monocytes. These cell types belong to the mononuclear phagocyte system (MPS), which originates in the bone marrow (BM). We observed that BM cells, a source of stem cells, were not susceptible to PRRSV. We could drive the BM cells into the MPS lineage by growing them in the presence of L929 cell supernatant as a crude source of macrophage colony-stimulating factor (M-CSF). The stimulated BM cells were characterized with monoclonal antibodies (MAbs) specific for cell markers such as MHC II, CD14, SWC1, SWC3, and SWC9, and with MAbs 517.2, 552.2, and PM18-7 that recognize an as yet unidentified macrophage marker. MAb 517.2 (specific for macrophages and a subset of monocytes) and MAb C4 that is directed to SWC9 (specific for macrophages) did not stain primary BM cells. However, between 2 to 4 days after culture, MAbs C4 and 517.2 started to stain a subset of stimulated BM cells. At the same time, some of these cells became susceptible to infection with PRRSV. The percentage of cultured cells that were stained by both MAbs and could be infected with PRRSV increased in time. These cells not only had a macrophage morphology, but also displayed macrophage functional properties such as phagocytosis of latex particles. The BM-derived macrophages that were cultured in the presence of M-CSF could be maintained for at least 3-4 months.

430 Rheumatoid Synovial Fibroblasts (RSF) and Human Monocytes Possess the Type V 14 kDa PLA₂

Lisa A. Marshall*, Elizabeth A. Capper, Amy Roshak, and Brian Bologne Department of Immunology, SmithKline Beecham Pharmaceuticals, King of Prussia, PA 19406

The release of arachidonic acid via activation of the phospholipase A₂ (PLA₂) family of enzymes is required for eicosanoid production by activated human monocyte and synovial fibroblast. Two isoforms of PLA₂ have been identified in the human monocyte, the Type IV 85 kDa and a 14 kDa isoform. We find the human monocyte does not have calcium-independent PLA₂. In the nonadherent P388D₁ macrophage and MMC-34 mast cell lines the 14 kDa enzyme has been identified as Type V PLA₂, a recently characterized isoform. We show, by RT-PCR, the 14 kDa PLA₂ found in the human monocyte is the Type V and not the Type IIA isoform. This was distinguished from the RSF where both Type IIA mRNAs were observed. Exposure of stimulated monocytes to the anti-phospholipase C antibody 3F10 did not inhibit LTC₄ production while the PLA₂ inhibitor, SB203347, inhibited LTC₄, but not PGE₂, formation. Analysis of the human monocyte showed the enzyme to be intracellular and not secreted. In contrast, RSFs were found to possess a cell surface associated PLA₂. Studies are currently underway to examine the subcellular localization of Type V PLA₂ in these cells.

432 Oxidative metabolism enzyme mRNA expression in monocyte-derived macrophages infected by HIV-1

Mialocq P., Clayette P., Dereuddre-Bosquet N., Martin, M., Dormont, D. CEA, SNV (DSV/DRM), CRSSA, IPSC, Fontenay aux Roses, France

Human immunodeficiency virus (HIV) causes a chronic ongoing inflammation and oxidative stress, particularly via macrophage lineage infection. These disorders are respectively characterized by high plasma levels of inflammatory cytokines and depletion of antioxidant molecule glutathione in HIV-infected patients. This cysteine-containing tripeptide is the major intracellular molecular defense against oxidative stress, an essential for several functions of cells. Its important role in HIV infection is confirmed by the link observed by LA. Herzenberg between glutathione deficiency and patient mortality [Proc Natl Acad Sci USA, 1997, 94, 11772]. Herein, we measured the mRNA expression of several enzymes involved in oxidative metabolism in human HIV-1/Ba-L-infected monocyte-derived macrophages (MDM). The mRNA expression of both superoxide dismutases (MnSOD and CuZnSOD), catalase, and glutathione peroxidase (GSH-Px) and reductase (GSH-Red) were measured all along the HIV-1/Ba-L replication kinetic in MDM using RT-PCR.

In uninfected or infected MDM, the catalase and CuZnSOD mRNA expression was not modulated during the culture. On the other hand, mRNA of MnSOD, GSH-Px and GSH-Red were transiently expressed: the MnSOD mRNA expression is followed by those of GSH-Px and GSH-Red (GSH-Px and GSH-Red: day 15 vs. MnSOD: day 12 of MDM culture). Moreover, HIV-1 infection of MDM did not significantly modify these mRNA expressions.

During monocyte differentiation into macrophages, an increase of tumor necrosis factor (TNF)- α synthesis was observed, and it is important to note that it precedes the mRNA expression of enzymes involved in oxidative stress. All together, these results suggest that i) GSH metabolism plays an important role in oxidative stress control, and ii) unlike TNF- α , HIV-1 infection did not interfere directly with the expression of oxidative metabolism enzymes.

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856.3

A NEW CLASS OF SELECTIVE INDUCERS OF THE MACROPHAGE INFLAMMATORY PROTEINS MIP-1 α AND MIP-1 β .

M.C. Bosco, A. Rapisarda, S. Massazza, and L. Varesio. Lab. of Mol. Biol., G. Gaslini Inst., 16147 Genova, Italy.

MIP-1 α and MIP-1 β (MIPs) are important inflammatory chemokines produced by activated macrophages. In this study, we demonstrate that the tryptophan catabolite picolinic acid (PA) and the iron chelator desferrioxamine (DFX) are potent inducers of MIPs mRNA expression in mouse macrophages in a dose- and time-dependent fashion and through a de novo protein synthesis-dependent process. MIPs induction was controlled at both transcriptional and posttranscriptional levels. The stimulatory effect of either agent was specific for MIPs, because other chemokines, including MCP-1, RANTES, and IP10, were not induced. Moreover, this response was stimulus-selective and not an epiphenomenon of macrophage activation, because IFN γ not only failed to induce MIPs expression, but inhibited the stimulation of both chemokines by PA or DFX. PA and DFX share the property of chelating iron, and we show that iron chelation was at least one of the mechanisms responsible for MIPs induction. In conclusion, we discovered a new pathway leading to inflammation initiated by tryptophan catabolism that can communicate with the immune system through the production of PA followed by secretion of chemokines by macrophages.

856.4

ICSBP CAN MEDIATE BOTH TRANSCRIPTIONAL ACTIVATION AND REPRESSION. S. Marecki and M. J. Fenton. Boston Univ. Sch. Med., Boston MA 02118.

Interferon consensus binding protein (ICSBP) is a member of the growing family of interferon regulatory factors (IRF). ICSBP is expressed in both lymphoid and myeloid cells, and has been reported to repress the expression of several interferon-responsive genes. ICSBP specifically binds the Ets-like protein PU.1 at composite IRF/Ets sites. Here we explore the ability of ICSBP and PU.1 to regulate macrophage genes. We found that ICSBP/PU.1 complexes can mediate transcriptional activation via IRF/Ets composite sites in macrophages. Furthermore, our data demonstrate that ICSBP can activate transcription of PU.1-dependent promoters that lack composite binding sites; specifically the scavenger receptor, IL-1 β , and CD11b promoters. In contrast, ICSBP can suppress transcription of the H-2L^d MHC class I promoter, a promoter which does not contain a PU.1 DNA binding motif. These data suggest that ICSBP can function in conjunction with PU.1 to activate the transcription of promoters containing either composite IRF/Ets sites or promoters containing only PU.1 sites. In contrast, ICSBP appears to repress promoters which lack PU.1 binding sites. Thus, the capacity of ICSBP to serve as either an activator or repressor of transcription may be dictated by other transcription factors with which it associates, such as PU.1.

856.5

FUNCTIONAL ASSOCIATION OF Fc ϵ R1 γ WITH ARGININE⁶³² OF PAIRED IMMUNOGLOBULIN RECEPTOR (PIR)-A3 IN MURINE MACROPHAGES. L.S. Taylor and D.W. McVicar (SPON: J. Ortaldo). Lab. Exp. Immunol., NCI-FCRDC, Frederick, MD 21702.

PIR are expressed on B cells and macrophages and include inhibitory and putative activating receptors referred to as PIR-B and PIR-A, respectively. Although PIR-B's inhibitory pathway has been described, it is unknown whether PIR-A receptors can deliver activation signals, and if so through what mechanism. Here we use chimeric receptors to address the mechanisms of PIR-A signaling in macrophages. Co-transfection of chimeric receptors comprised of the extracellular region of human CD4 and the transmembrane and cytoplasmic domains of murine PIR-A3 demonstrated the ability of PIR-A3 to physically interact of the Fc ϵ R1 γ chain in 293T cells. This interaction is dependent on Arg⁶³² within the PIR-A3 transmembrane domain. In addition, we demonstrate PIR-A3 interaction with the endogenous Fc ϵ R1 γ of the ANA-1 macrophage cell line, again in an Arg⁶³²-dependent manner. Furthermore, we show that crosslinking of these chimeric receptors synergizes with IFN- γ in the production of nitric oxide. These findings are the first to demonstrate the potential of PIR-A3 to deliver activation signals to macrophages and establish its dependence on Arg⁶³². These findings suggest that further study of the PIR-A receptors should be aggressively pursued toward a complete understanding of the intricate regulation of macrophage biology.

856.6

PROTEIN KINASE CK2 ACTIVATION IS MEDIATED BY THE MAP KINASE PATHWAY IN LPS-STIMULATED MACROPHAGES. T. K. Means and M. J. Fenton. Boston Univ. Sch. Med., Boston MA 02118.

Stimulation of macrophages by Gram-negative lipopolysaccharide (LPS) rapidly leads to the activation of several protein kinases and the subsequent phosphorylation of transcription factors that regulate cytokine genes. We previously reported that LPS stimulation also up-regulates the enzymatic activity of protein kinase CK2, although the signaling cascade that leads to CK2 activation is unknown. We performed additional studies in order to determine if CK2 activation was a consequence of MAP kinase (MAPK) activation. Our studies revealed that CK2 activity was rapidly and transiently up-regulated in LPS-stimulated RAW264.7 macrophages. We found that PD98059, an inhibitor of Erk kinase activation by the MAPK kinase MEK-1, blocked LPS-induced up-regulation of CK2 activity. In contrast, the p38 kinase inhibitor SB203580 did not block CK2 activation by LPS. This finding suggests that CK2 activation was mediated by the Erk kinase signaling cascade, but not by the p38 kinase cascade. We also found that PD98059 blocked LPS-induced serine phosphorylation of PU.1, an Ets-like transcription factor that is a substrate for CK2. In transient transfection analyses, the MEK inhibitor selectively blocked LPS-inducible activation of the PU.1-dependent CD11b promoter. These studies demonstrate that activation of CK2 and PU.1 appears to require the Erk signaling cascade in LPS-stimulated macrophages.

856.7

THE NEUROPEPTIDES VIP AND PACAP INHIBIT NITRIC OXIDE PRODUCTION BY LPS-ACTIVATED MACROPHAGES. M. Delgado, E.J. Munoz-Elias, R.P. Gomez and D. Ganea. Rutgers University, Newark, NJ 07102 and Universidad Complutense, Madrid, Spain.

High output nitric oxide (NO) production from activated macrophages represents a major mechanism for macrophage cytotoxicity against pathogens. However, despite its beneficial role in host defence, sustained high output NO production is also implicated in a variety of acute inflammatory diseases. Therefore, the downregulation of iNOS expression during an inflammatory process plays a significant physiological role. This study examines the role of two immunomodulatory neuropeptides, VIP and PACAP, on NO production by LPS-, IFN γ -, and LPS/IFN γ -stimulated peritoneal macrophages and the Raw 264.7 cell line. Both VIP and PACAP inhibit NO production in a dose- and time-dependent manner. The inhibitory effect is partially mediated through increases in IL-10, and independent from other macrophage-secreted cytokines, including TNF α . The VIP/PACAP receptors VPAC1, which is constitutively expressed in macrophages, and to a lesser degree VPAC2, which is induced upon macrophage activation, mediate the effect of VIP/PACAP. VIP/PACAP inhibit iNOS expression and activity both in vivo and in vitro. Two transduction pathways appear to be involved, a cAMP-dependent pathway which preferentially inhibits IRF-1 transactivation, and a cAMP-independent pathway which blocks NF κ B binding to the iNOS promoter. The downregulation of iNOS expression, together with previously reported inhibitory effects on the production of the proinflammatory cytokines IL-6 and TNF α , and the stimulation of the anti-inflammatory cytokine IL-10, define VIP and PACAP as "macrophage deactivating factors" with significant physiological relevance.

Grants: PHS AI 41786-01 (DG); Busch Biomedical Award 96-98 (DG), PB94-0310 (RPG), and Postdoctoral Fellowship from the Spanish Department of Education and Science (MD).

PRESENTATION AT ERA OF HOPE MEETING 1997

EXTENDED ABSTRACT (IN MIDDLE BOX)

**BREAST CANCER RESEARCH
TRAINING PROGRAM**

**Adrianne E. Rogers, M.D.^{PS}
and Theodore Colton, Sc.D.**

**Boston University School of Medicine
& School of Public Health**

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The purpose of the program is to train predoctoral students at Boston University Schools of Medicine and Public Health (BUSM, BUSPH) to perform research relevant to the etiology, prevention, detection, diagnosis and therapy of breast cancer using the most advanced knowledge and techniques available. In addition to providing in-depth training in the student's chosen discipline, the purpose of this training program is to assure her or his education in other relevant disciplines in order to gain an understanding of the contribution of these disciplines to breast cancer research and to apply their basic concepts and methods in breast cancer research. Emphasis is placed on interdisciplinary training in Pathology, Epidemiology, and Cell and Molecular Biology. The goal is that, upon completion of the degree in a particular discipline, trainees will be able to work and communicate effectively with other scientists in interdisciplinary research focused on breast cancer.

Eight students are being trained in an interdepartmental curriculum utilizing courses, seminars and research mentors in the disciplines listed. Mentors are selected from faculty whose research is in or highly relevant to breast cancer. Examination and dissertation committees are composed of mentors and other faculty in the disciplines listed. The interdisciplinary structure of the program is further promoted by special seminars in conjunction with the Program in Research on Women's Health (PRWH).

Two trainees per year have been selected from applicants to the doctoral programs in Pathology or Biostatistics and Epidemiology on the basis of grade point average, Graduate

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Keywords: Predoctoral Training, Interdisciplinary Program, Breast Cancer Research Training

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-94-J-4421

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Record Examination scores, letters of recommendation, telephone or personal interview, demonstrated ability in and commitment to research, and strength of interest in breast cancer research. This nucleus of students has expanded to include six additional graduate students in Pathology or Biochemistry who are working in laboratories with the trainees and are supported by other funds.

The institution has contributed significant stipend, tuition, research and administrative financial support to the program and has benefited by attracting and retaining high quality students and by the addition of seminars and research collaborations focused on breast cancer research. Of the eight students, two entered with significant experience in experimental and clinical breast cancer research, one of them with a public health degree (MPH) also; two students entered after two years of medical school, one with a bachelor's degree and extensive clinical laboratory experience and an MPH, and three entered after completing bachelor's degree science programs.

The interdisciplinary focus has been established and maintained by:

- 1) the requirement that students in Pathology take an epidemiology methods course and one advanced epidemiology course (cancer prevention, environmental epidemiology, cancer epidemiology or genetic epidemiology) and that students in Biostatistics and Epidemiology take Basic and Experimental Pathology and attend the weekly Pathology research seminars.
- 2) participation of all students in ongoing studies in the 7,12-dimethylbenzanthracene (DMBA)-induced breast cancer model.
- 3) student and faculty discussion and statistical analysis of specific results from students' research.
- 4) seminars and working group meetings organized by the program, the departments, the PRWH and the Cancer Prevention Program, all at the Medical Center. Students have luncheon discussions with seminar speakers invited by the program.
- 5) participation in the annual Russek Graduate Student Achievement Day at the Medical Center.
- 6) seminars in the wider biomedical community, notably the Mass. Dept. of Public Health Breast Cancer Research Lecture Series and at other research institutions.

The first two students in the program are fully engaged in their research: one investigating dietary interactions with steroid hormone receptor responses in DMBA- induced mammary gland tumorigenesis in rats; one investigating the influences of environmental and lifestyle factors on cancer risk in a large cohort of African-American women. The others are preparing for qualifying examinations, taking courses and continuing or beginning research. Their potential research projects include: cytokines and cellular immunological responses in transgenic mouse models of breast cancer, vitamin D and breast cancer, responses of mammary gland nuclear transcription factors to DMBA and to dietary factors that influence tumorigenesis, epidemiological study of breast cancer survival, risk factors and tumor steroid receptors, development of novel analytical techniques for assessing risk of breast cancer using a dataset of over 1,000 pedigrees..

The students who are sufficiently advanced in their research are preparing grant applications to several funding sources. The more senior students participated in a formal course in grant-writing at the beginning of their studies.

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**TITLE BREAST CANCER (RESEARCH APS)
TRAINING PROGRAM**
**Adrienne E. Rogers, MD (ps)
and Theodore Colton, Sc.D.**
**Boston University School of Medicine
& School of Public Health**

LAY/PUBLIC ABSTRACT: SINGLE SPACE 250-WORD ABSTRACT BELOW THIS LINE. PLEASE LEAVE 1" SPACE BETWEEN AFFILIATION AND BEGINNING OF ABSTRACT TEXT. DO NOT EXCEED ONE PAGE.

Research into the causes, prevention and treatment of breast cancer requires work in many scientific disciplines. Investigators trained in one discipline may find it difficult to understand related work of investigators in other disciplines and to establish collaborative studies that might be important. The training program was established to teach PhD and MD-PhD students basic knowledge and elements of research in Pathology, Cell and Molecular Biology, and Biostatistics and Epidemiology. These disciplines are used extensively in breast cancer research. Eight students are in the program, which is supported jointly by the grant and Boston University. They have participated in joint seminars, courses and a research project in diet and breast cancer in laboratory rats. The more advanced students are now beginning their own research projects.

The program is important to breast cancer research because it is producing scientists trained to carry out investigations that may include two or more research disciplines that complement each other.

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INVESTIGATOR

LAY ABSTRACT

Research into the causes, prevention and treatment of breast cancer requires work in many scientific disciplines. Investigators trained in one discipline may find it difficult to understand related work of investigators in other disciplines and to establish collaborative studies that might be important. The training program was established to teach PhD and MD-PhD students basic knowledge and elements of research in Pathology, Cell and Molecular Biology, and Biostatistics and Epidemiology. These disciplines are used extensively in breast cancer research. Eight students are in the program, which is supported jointly by the grant and Boston University. They have participated in joint seminars, courses and a research project in diet and breast cancer in laboratory rats. The more advanced students are now beginning their own research projects.

The program is important to breast cancer research because it is producing scientists trained to carry out investigations that may include two or more research disciplines that complement each other.

INTRODUCTION

Information about breast cancer is increasing rapidly but is incomplete and often inconsistent and controversial because of the complexity of the disease and its causes. Increased integration of knowledge and research results among investigators from different scientific disciplines should enhance progress in understanding this disease. This Training Program was designed to coordinate the training of doctoral students in Pathology and in Biostatistics and Epidemiology to conduct interdisciplinary research on breast cancer.

The **PURPOSES** of the Program are to:

- 1) Promote the development of young investigators by giving them a multidisciplinary background in breast cancer biology and epidemiology in addition to intensive training in a specialized research area.
- 2) Establish and carry out a multidisciplinary academic research training program in breast cancer biology and epidemiology.
- 3) Produce graduates in one discipline (Pathology, Epidemiology) who have an understanding of the other discipline and who can perform collaborative, multidisciplinary research in the etiology, prevention and therapy of cancer.
- 4) Provide training in cell and molecular biology, experimental pathology, carcinogenesis, epidemiology, biostatistics, immunology, toxicology and nutrition that will permit trainees to explore: a) basic breast cancer cell processes and interactions; and b) questions about etiology, prevention and therapy of breast cancer; and to integrate knowledge derived from the different approaches.
- 5) Increase collaborative research in breast cancer among faculty and trainees.

PROGRAM

Predoctoral students interested in breast cancer research, admitted to the Departments of Pathology and Laboratory Medicine or Biostatistics and Epidemiology, were selected for the program on the basis of GPA, GRE scores and letters of recommendation. The Trainees follow a curriculum that includes courses in pathology, epidemiology and biostatistics in addition to the required courses in their fields of study. They participate in ongoing studies of dietary effects on DMBA-induced mammary gland carcinogenesis in rats.

After passing the qualifying examination, each Trainee chooses a dissertation advisory committee of faculty with expertise in or closely related to breast cancer research. Trainees present their research results on a regular basis to their committees and ultimately defend their dissertation before the committee and other faculty and students.

Trainees participate actively in seminars and research group meetings at BUMC and in local, regional and national meetings. They are trained in grant writing and encouraged to submit applications to appropriate funding sources.

The Trainees are closely integrated into the Breast Cancer Working Group, organized through the Program in Research on Women's Health, in which basic science, clinical and epidemiology faculty meet regularly to discuss research questions and results.

TRAINEES

There are 8 Trainees in the Program, two admitted per year beginning in 1994.

The eight students are:

1994: Yvette Cozier (BA, Liberal Arts, Harvard Extension School, 1987; MPH, BUSPH, 1994) was admitted to the Biostatistics and Epidemiology program. She had extensive laboratory experience in clinical hematology and microbiology. Her research, in Dr. Lynn Rosenberg's Black Women's Health Study, is focused on breast cancer risk factors and prevention in that population.

Laurie Hafer (BS, Microbiology, Penn State Univ., 1989) was admitted to the Pathology and Laboratory Medicine program. She had significant experience in development of immunohistochemical research methods focused on breast cancer. Her dissertation research title is "Black tea and estrogen and progesterone receptors in the rat mammary gland".

1995: Sylvia Marecki (BS, Microbiology, Univ. N.H., 1995) was admitted to the Pathology and Laboratory Medicine program in the immunology track. She had undergraduate research experience in microbiology and had been awarded two competitive research grants. Her dissertation research is in the regulation of inducible macrophage gene expression at the level of gene transcription.

Paul Johansen (BS, Biology, Yale, 1988) was admitted to the Biostatistics and Epidemiology program after completing 1 1/2 years of medical school to pursue interests in biomedical applications of mathematics and statistics. He is beginning his dissertation research which is entitled: "Breast and ovarian cancer risk assessment following prophylactic surgery: A comparison of statistical models using BRCA1 and BRCA2 pedigree data."

TRAINEES (CONTINUED)

1996: Jackie Ashba (BA, Biology & Economics, Clark University, 1989; MPH, BUSPH, 1992; MA, Medical Sciences, BUSM, 1994) was admitted to Biostatistics and the Epidemiology program after completing Masters degree research on hormone receptor phenotypes in breast cancer.

Ingrid Gherson (BS, Biology, Binghamton University SUNY, 1996) was admitted to the Pathology and Laboratory Medicine program. She had significant technical histopathology laboratory experience. She has begun preparatory dissertation research on the effects of vitamin D analogues on proliferation of breast cancer cells.

1997: Kathryn Kavanagh(MD-PhD student at BUSM, admitted from the Royal College of Surgeons, Dublin), is in the Pathology and Laboratory Medicine program. She spent a year in molecular biology research at Albert Einstein College of Medicine and has begun dissertation research studies of the inhibitor of NF-KB, I κ B- α , in breast cancer.

Elizabeth Jiyoung Lee(BS, Microbiology and Genetics, UCLA) was admitted to the Pathology and Laboratory Medicine program. She had excellent undergraduate research experience in studies in laboratory rodents.

CONCLUSIONS

The Program has successfully recruited and retained excellent Trainees from diverse backgrounds to focus on breast cancer research. The interdisciplinary focus is strong and is fostered by the required epidemiology and pathology courses, seminars and the frequent, stimulating and valuable interactions with faculty and other students in the Breast Cancer Working Group. There are productive discussions of the performance of and data from the DMBA projects that directly foster the interdisciplinary goals of the program. All Trainees whose work is sufficiently advanced have submitted applications for research grants to the DOD program and the Massachusetts Breast Cancer Research Program.

Considerations in the design of studies of dietary influences on mammary carcinogenesis in rats and mice

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Key words: breast cancer, carcinogenesis, diet, mammary tumors

Summary

Design of diets for the study of dietary influences in mammary gland carcinogenesis requires attention to several questions: (1) Do the diets satisfy the nutritional needs of the animal under the conditions of the experiment, and are they palatable? (2) Does the protocol include determination of feed intake (if indicated) and of achievement of the desired level of nutrient deficiency, adequacy, or excess? (3) Are there potentially confounding nutrient interactions or nutrient effects or physiological or pathological responses that must be considered?

The particular sensitivity of mammary gland tumorigenesis to intake of fat and calories and to body weight gain must be considered and controlled for in all experiments.

Introduction

Many of the papers in this volume present information on diets for laboratory rodents used in the investigation of the effects of specific dietary components on mammary gland carcinogenesis. The authors present experimental results related to the supply, utilization, and effects of the specific dietary components investigated. There are certain considerations in diet design that are general to virtually all such studies and other considerations that are more specifically dictated by the component(s) being studied. General and some specific considerations will be discussed here, primarily using studies in rats.

General questions to be asked about design of experiment protocols and diets for investigation of

dietary and nutritional effects on mammary carcinogenesis studies are:

1. Do all diets to be compared supply all the essential nutrients (or all except the nutrient to be studied) in amounts and forms that are known to be adequate to support normal growth, development, and maintenance in animals of the species, strain, and gender to be used in the experiment?
2. Have changing nutrient needs of the animals due to physiological, toxicological, infectious or other challenges in the experiment been considered?
3. Does the protocol include assays to determine that deficiency or excess of the dietary component(s) to be studied has, in fact, been achieved?
4. Are the diets known to be palatable to the

animals to be used?

5. Can the diets be formulated so they can be fed in the form and with the frequency desired to provide the animals with palatable feed containing the expected nutrient amounts at all times or as needed for the experiment?

6. Do the diets and methods of feeding permit ready and accurate measurement of feed intake if the protocol requires it?

7. Are there potentially confounding nutrient interactions or effects of the diets on ingestion, absorption, metabolism, distribution, uptake, or excretion of nutrients, carcinogens, or other endogenous or exogenous chemicals?

8. Are there potentially confounding effects of the diets on tissue or organ function, cell growth, division, lifespan, or repair, or on responses to immunological or other stimuli in the tissues of interest?

Each of these questions is discussed briefly.

1. Essential nutrients for animals to be used

A recent monograph gives detailed information on nutrient requirements of rats, mice, and other laboratory animals, and provides valuable discussions of diet formulation [1]. Diet formulations that meet the known nutrient requirements of laboratory rats and mice are readily available in the literature [1-5], and there are several reliable commercial sources for the diets and for individual components. Formulation of diets in each laboratory requires knowledge of the nutrient requirements of the animal to be used and careful attention to the effects on nutrient intake of varying the composition of the diet or the conditions of the experiment. Because rodents tend to adjust feed intake to keep energy balance reasonably constant, they eat smaller amounts of energy dense diets than of less energy dense diets. Therefore diets should be designed to supply protein and all micronutrients in the recommended amounts on an energy basis rather than on a weight basis [1-4,6]. Nutrients should not be

supplied in amounts significantly in excess of the requirement, unless the excess is the subject of study, since many of them have anticarcinogenic activity, and some may enhance carcinogenesis under certain conditions [7].

Investigators in the National Toxicology Program (NTP) have published findings in large groups of Fischer 344 (F344) rats and B₆C₃F₁ mice that served as controls for chemically treated animals in long-term studies [8-11]. In a comparison of several unrefined diets with approximately equal caloric density, they found that, compared to the standard open formula diet in use at NTP (the NIH-07 diet), diets that contained by weight less protein (15 vs 23%), more crude fiber (9-14% vs 4%) and more fat (7-8% vs 5% polyunsaturated oils) supported somewhat increased survival, decreased body weight, and decreased burden of spontaneous mammary gland tumors in rats. The major impetus for the study was the problem of decreased survival due to nephropathy in male F344 rats; this condition was reduced in both males and females by the dietary changes. Based on this work, a new unrefined diet has been developed for use at the NTP [5].

Purified diets have been developed to meet certain objections to the AIN-76 A diet that is widely used. In the committee publications [3,4] the diets are described; the rationale is given for choice of and changes in ingredients, and the life stage for which they are designed is given. Guidelines are given for alteration of the diets if required by the experimental protocol. The guidelines include: increasing the content of antioxidants if the polyunsaturated fats are increased, changing phosphorus reciprocally with casein and in general reformulating the mineral mix if the protein source is changed, using a standardized fiber source that does not contain large or variable amounts of essential or toxic minerals, and reformulating on a caloric rather than a weight basis to assure adequate intake of essential nutrients. Users of modifications of the diets are cautioned to check also the effect on composition and pelleting of the sugar vehicle used for the vitamins, and the N-6:N-3 fatty acid ratio and the

polyunsaturated:saturated fatty acid ratio of all the vegetable oils used since commercial hydrogenation or changes in the cultivar can change fatty acid composition [3,4].

2. Changing needs

Changing nutritional needs, particularly for energy and protein, with life stage and growth rate and the effect of these changing needs on toxicological or other responses are a subject of research and debate [12-14]. Pregnancy and lactation are physiological considerations of major importance in designing diets for mammary tumorigenesis studies [1]. Because of the large nutrient and energy demands of pregnancy and lactation, rats may be unable to compensate adequately for reduction of nutrient density, for example, by addition of fiber to their diet.

In designing control groups, investigators must anticipate effects on feed intake, nutrient utilization, and body weight gain of intentional nutrient deficiency or excess, of carcinogens, or of test substances such as ethanol, tea, coffee, and hosts of other natural products or chemicals [15].

As is evident from several papers in this volume, energy intake, fat intake, and body weight are major determinants of spontaneous or carcinogen-induced mammary tumorigenesis. This is true of tumorigenesis in other tissues and organs as well, but has been most extensively documented and discussed in mammary gland tumorigenesis [7,14,16,17]. If measures are not taken to assure comparability of body weight gain between or among groups, tumorigenesis results may be uninterpretable. The lower limit of the significant weight difference is not precisely known, but it appears that any difference greater than 10% must be viewed as possibly contributing to differences observed in tumorigenesis end points [14].

Use of pair-feeding, based on feed intake or on body weight, may be necessary to meet this problem; pair-feeding is a more effective control when applied to individually-paired than to group-

paired animals.

There are many examples of interactions between chemicals, including drugs, and nutritional status. One such interaction relevant to mammary carcinogenesis is interference of the chemotherapeutic agent procarbazine with choline metabolism, resulting in both exacerbation of dietary choline deficiency and increased mammary gland carcinogenesis in rats [18,19].

3. Confirmation of planned deficiency or excess

Assay of tissue content or biological activity or histological changes related to the dietary component(s) of interest should be made. For many nutrients there are well-standardized functional or chemical assays [1,20]. The results are most meaningful if the assays are performed on the tissue that is the focus of the carcinogenesis study as well as on the tissues generally used to study the nutrient. Measurement of deficiency or excess of nutrient or other diet components in the tissue(s) of interest may mean a simple chemical or biochemical assay or a much more complex evaluation, for example assay of fatty acid composition of membranes or of products of oxidative damage. The assay chosen may be determined by the hypothesis being tested.

4. Palatability

Palatability of diets usually can be readily assessed in a pilot 2-3 week study comparing feed intake and weight gain of young animals fed a new diet compared to animals fed a diet known to be nutritionally adequate and palatable.

5. Formulation considerations

The formulation of diets requires attention to:

- a) the stability of nutrients and any propensity of diets to grow mold at the room and refrigerator temperatures at which they will be stored and fed. These considerations dictate the frequency with which diets must be mixed and fed and the conditions under which they must be stored.
- b) protocol requirements for specific components, such as fats that are liquid or very soft at room temperature or ethanol. Soft or liquid fats may necessitate hardening of the diet by incorporating it into a gel or liquefying the diet and possibly including emulsifiers. Feeding ethanol requires a liquid diet as well as provision for adequate water intake [21].

Proper preparation and storage of diets and ingredients are critical to maintaining consistent composition and palatability [1,22]. Commercial suppliers of diets or ingredients should provide appropriate analyses, or the investigator should make independent analyses. Vitamins can be destroyed by light and heat; mineral salts, choline compounds and other components may be hygroscopic and distort weight measurements; fats may be readily oxidized at room temperature. Vitamins and minerals are generally prepared as premixes in sucrose or other convenient vehicles so that they can be added in accurately measured amounts to the diet. Vitamin premixes can be stored in the dark and protected from the air at -20°C – -80°C for up to 6 months. Minerals or mineral mixes may require grinding and should be stored in a dessicator. Both premixes and the final diet must be thoroughly mixed, but not over-mixed which can cause heating and oxidation [1].

Requirements for storage of prepared diets vary with composition. The basic AIN diets and similar diets can be stored tightly closed at 4°C for about 3 months or frozen for about 6 months. If diets are formulated without antioxidants or if they are in liquid or gel form, they can be stored refrigerated for about one week because of mold or bacterial growth. Unrefined diets can generally be stored in air-conditioned rooms for up to six months [1].

6. Feed measurement requirements

If there are protocol requirements for measurement of feed intake, animals must be housed individually, and diet form and feeders must permit easy measurement of feed given and recovery of feed not consumed. Firm pellets or gel blocks are usually easily handled, but crumbling of them by the animals may require special feeders.

7. Nutrient interactions

Nutrient interactions and tissue storage, gastrointestinal absorption, metabolic adjustments, and changes in growth rate and nutrient utilization all may inhibit or enhance the achievement of the desired nutritional condition of the animals. Induction of certain nutrient deficiencies, for example vitamin A, may require the deriving of offspring from deficient dams to reduce body stores. Nutrients may interact antagonistically (for example, vitamins A and E and carotenoids or vitamin K, certain amino acids, polyunsaturated fatty acids and selenium or other antioxidant nutrients, ascorbic acid and selenium), or they may spare one another (for example, the lipotropic factors, choline, methionine and folate or selenium and vitamin E) [1]. Absorption of nutrients and test compounds may be influenced by the composition or form of the diet; many examples are listed in a recent monograph [1]. Under this question and others as well, come considerations of the form, identity, and purity of diet ingredients. Rodents fed cereal-based, unrefined (hereafter referred to as unrefined) diets tend to manifest reduced chemical carcinogenesis in the mammary gland and other target organs compared to rodents fed semipurified diets [7,15]. The major endpoints of mammary tumorigenesis (tumor latency, incidence, and multiplicity) in the DMBA-or MNU-induced models vary from experiment to experiment within and among laboratories [23-26], which makes it difficult to compare results of different experiments. There

are few direct comparisons reported of mammary gland carcinogenesis in rats fed the two kinds of diet. In direct comparisons that are available [25] and in sequential experiments in the same laboratory [24,26], a moderate carcinogenesis-inhibiting effect of unrefined diets compared to semipurified diets is shown.

There are many possible explanations for this difference; they include the content of anti-oxidants, Phase I and II enzyme inducers, high and variable amounts of nutrient and non-nutrient anticarcinogens and of fiber, and the presence of ultratrace elements in the unrefined diets [1,7,15, 28,29]. Unrefined diets are not appropriate for use in studies of nutrient effects on carcinogenesis because of the variable nutrient content that arises from variations in the natural products themselves and from the addition and the degradation with time of nutrients, particularly vitamins [1]. Even in studies of excessive supplementation with a single nutrient, variable quantities of other nutrients and of non-nutrient substances that may influence carcinogenesis make unrefined diets unsuitable. Contaminants (heavy metals, mycotoxins, nitrosamines, phytoestrogens) in unrefined diets can, of course, have profound effects on experimental results. Diets assayed for the major contaminants are commercially available.

8. Confounding physiological effects

Diets deficient in one or more nutrients can increase or decrease cell, tissue, and organ function. While deficiencies generally reduce cell growth and turnover, they can markedly increase cell growth, turnover, and number. Examples include the effect of lipotrope deficiency on hepatocytes and of zinc deficiency on esophageal epithelium [19,30].

Nutrients can be toxic and, if added in excess to the diet, may have non-specific effects on carcinogenesis because of their toxicity. The fat-soluble vitamins, minerals, amino acids, and protein all can cause specific toxicities as well as reduced feed intake and other general effects [1].

Other considerations

Dietary effects on mammary tumorigenesis can be readily demonstrated using a variety of models and protocols; strong effects include the enhancing effects of diets high in N-6 polyunsaturated fats and in energy density [7,14,16,17, and this volume]. Other effects are relatively weak or variable and may be demonstrable only under certain conditions; these include the variably enhancing effects of high protein diets and, to some extent, the anticarcinogenic effects of selenium and of certain non-nutrients [7]. Investigation of weak or variable effects may be aided by use of low doses of carcinogen to reduce the carcinogenic stimulus and lengthen the latency period. This approach has drawbacks since it may increase the ratio of benign to malignant tumors and also increase the likelihood of appearance of spontaneous mammary tumors; it adds to the cost of the experiment by lengthening its duration. However, it may increase the sensitivity of the bioassay.

The choice of models is governed by knowledge of their responses related to the questions to be asked, by their availability, cost and ease of handling, and by the investigator's experience. By far the greatest amount of information on diet and experimental mammary carcinogenesis has been obtained from the DMBA- or MNU-treated female Sprague-Dawley rat. A recent extensive review of these tumor models is available [31]. Studies of spontaneous carcinogenesis in this rat and of spontaneous or chemically-induced carcinogenesis in F344 rats and several strains of mice have supplied additional or confirmatory evidence. There is a marked contrast between Sprague-Dawley and F344 rats in aging of the endocrine system [32]; however, major differences are not reported between the two strains in dietary effects on mammary carcinogenesis [23, 33]. The absence of a difference may be the result of the fact that rats are exposed to the carcinogen and begin to develop tumors early in their lifespan, before the endocrine changes of aging have been established. The endocrine

changes with aging are reduced in Sprague-Dawley rats that are severely (50%) but not moderately (25%) restricted in feed supplied [13].

The mouse models have been used much less extensively than rat models in dietary studies. In the NTP studies of control mice referred to above [9-10], liver tumors were the only tumors strongly correlated with body weight in females; the high incidence of spontaneous liver tumors in many mouse strains is a significant deterrent to their use in studies of carcinogenesis at other sites. However the availability of genetically altered mice for mammary carcinogenesis studies and the advantages they offer in investigation of many questions means that dietary studies in them will be of increasing interest. The nutritional needs of mice are fully discussed in the NRC monograph [1]. If specific models require restricted or absent intestinal flora, their nutritional needs may be altered because the flora contribute significantly to the supply and metabolism of many nutrients. In addition, the diets must be fortified to allow for nutrients lost through sterilizing procedures [1].

As many as possible of the endpoints of carcinogenesis in the mammary gland should be used to evaluate dietary influences since the influences may be relatively weak. The endpoints are not, of course, independent, but they do magnify effects. For example, a group of rats with a short average latency to first tumor is likely to show at necropsy higher incidence, a greater number and volume of tumors per rat, and a greater proportion of malignant tumors than a group of rats with a longer average latency to first tumor. The differences in endpoints may vary in statistical significance but be, in aggregate, convincing. Sophisticated and sensitive statistical analyses of these kinds of data are discussed in this publication. They offer methods to derive information from data that may initially appear uninformative, methods for combining data from more than one experiment, and methods for increasing comparability of data from different experiments.

A problem related to diet and mammary gland carcinogenesis that is not discussed in this

publication is the question of an influence of ethanol. Ethanol has many toxicological and nutritional interactions that are potentially important in carcinogenesis in the mammary gland and other target tissues. Epidemiological data indicate an increase in breast cancer risk in women associated with even modest alcohol use [34]. Studies in laboratory rodents have not yielded consistent results [21,35]. Diet design for studies of ethanol effects must take into account the calories supplied by ethanol and the effects of ethanol on fluid balance. Rodents will not voluntarily ingest ethanol mixed in their drinking water so it must be given in a liquid diet which they will ingest or by gavage. There are diets published that are nutritionally balanced for different alcohol and fat contents [21,35,36]. Diets are available commercially; they should be carefully evaluated for nutritional adequacy and for comparability of ethanol-containing and control diets.

Publications should always specify the diets(s) fed and give the composition or refer to a recent, readily accessible publication of the composition. Any modification of the published composition must, of course, be clearly presented.

In all tumorigenesis studies, and particularly in mammary tumorigenesis studies, assessment of feed intake and body weight gain throughout the study should be made and reported. Initial and final body weights are not sufficient because weights tend to plateau, more in female than in male rats and markedly in mice, and critical differences during tumorigenesis may disappear or be reduced by the end of the experiment. If weights do not differ significantly among groups, feed intake can be assumed to be comparable and need not be measured. However, if the animals are given substances such as coffee or tea or fed diets of differing nutrient density or absorbability that may alter appetite and nutrient or energy utilization, intake should be measured.

Acknowledgments

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